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Lasers in Surgery and Medicine 44:856–864 (2012)

Blue LED Inhibits the Growth of *Porphyromonas gingivalis* by Suppressing the Expression of Genes Associated with DNA Replication and Cell Division

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Background and Objectives: Blue light has been employed or investigated in both the medical and dental fields. Many studies have so far been reported a bactericidal effect of blue light emitting diodes (LED). However, it is still unclear whether exposure to blue LED kills or inhibits the growth of bacteria. We therefore investigated the effect of blue LED irradiation on the growth of *Porphyromonas gingivalis* compared with the effects of red LED.

Materials and Methods: *P. gingivalis* cell suspensions were irradiated with blue or red LED (135 J/cm^2) anaerobically, incubated for various lengths of time, and then the total RNAs were isolated. The RNA degradation and gene expression levels of stress-related proteins in blue or red LED-irradiated samples were examined using the RNA integrity number (RIN) and RT-PCR, respectively. Quantitative RT-PCR was done to investigate the gene expression profiles associated with chromosome replication and cell division.

Results: Exposure to blue LED delayed the growth of *P. gingivalis*, while red LED did not. The RIN value indicated no RNA degradation in either the blue or red LED-irradiated samples. In addition, the gene expression levels of stress-related molecules remained either constant or increased 15 minutes after the blue LED irradiation compared to that before irradiation, thus suggesting that blue LED may not kill *P. gingivalis* cells. However, the blue LED irradiation did lead to a remarkably decreased expression of genes associated with chromosomal DNA replication and cell division after 5 minutes; exposure to the red LED did not.

Conclusion: The inhibition of the growth of *P. gingivalis* by blue LED may therefore be induced not by a bactericidal effect, but instead due to a bacteriostatic effect mediated by the suppression of the genes associated with chromosomal DNA replication and cell division at the transcriptional level. Lasers Surg. Med. 44:856–864, 2012. © 2012 Wiley Periodicals, Inc.

Key words: cell division; DNA replication; gene expression; growth; LED; *Porphyromonas gingivalis*

INTRODUCTION

Subgingival plaque biofilm microbiota in periodontal pockets are thought to be associated with the development and progression of periodontitis [1–3]. *Porphromonas gingivalis* is a black-pigmented, Gram-negative anaerobic bacterium, which is thought to be an important etiological agent in adult periodontal disease [4–6] and it possesses many virulence factors, such as fimbriae, proteases, hemagglutinins, and capsular polysaccharide, that allow this organism to cause disease [4]. This bacterium is known to mediate the coaggregation of various oral Gram-positive and Gram-negative bacteria. In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilms containing multiple bacterial species, which colonize specific surfaces and result in interactive nutritional chains. Therefore, the regulation of this bacterium may lead to the regulation of the

Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest and none were reported.

Contract grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Contract grant sponsor: Japan Society for the Promotion of Science (JSPS); Contract grant number: 22592308, 21390497.

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Accepted 1 October 2012
Published online 5 November 2012 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/lsm.22090

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biofilms, and it may thus be useful for improving or maintaining gingival health.

Recently, light and light emitting diodes (LED) have been introduced into the field of medicine and dentistry; they have been employed or investigated as treatments for neonatal hyperbilirubinemia [7,8], gastric ulcers caused by *Helicobacter pylori* [9], various skin diseases [10–13], wound healing [14], and oral bacterial elimination [15–17]. In addition, there have been many reports about the effects of LED on bacteria. Red light or near infrared light was reported to exert no antimicrobial effects [18–20]. In contrast, blue light has been reported to possess a bactericidal effect; the delivery of blue light (representing 15–58.8 J/cm^2 energy density) is sufficient to reduce the viability of bacteria such as *H. pylori* [9], methicillin-resistant *Staphylococcus aureus* [21], *Pseudomonas aeruginosa*, and *S. aureus* [22]. Regarding periodontal pathogens, blue light/blue LED (4.2–94 J/cm^2) reduce the survival rates of *P. gingivalis* [20], *Fusobacterium nucleatum* [20], *Prevotella internedia* [23], and *Prevotella nigrescens* [23]. Upon the blue LED irradiation, it has been speculated that endogeneous prophyrin produced by bacteria is excited and leads to photodynamic reaction through the singlet oxygen production, thus resulting in bacterial killing [24]. However, it is still unclear whether exposure to blue LED kills or inhibits the growth of bacteria. In addition, little is known about the molecular basis regarding the effects of LED irradiation on bacteria. In this study, we therefore investigated the effect of blue LED irradiation under anaerobic conditions on the growth of *P. gingivalis* compared with the effects of red LED and the gene expression profiles of *P. gingivalis* after blue and red LED irradiation in order to obtain a better understanding of the mechanism(s) underlying the putative growth inhibitory effect.

MATERIALS AND METHODS

Bacteria and Culture Conditions

P. gingivalis ATCC 33277^T was grown in tripticase soy broth supplemented with phytone peptone (5 mg/ml), yeast extract (5 mg/ml), NaCl (5 mg/ml), cysteine (0.1%), hemin (5 μg/ml), menadione (1 μg/ml), and kanamycin (50 μg/ml) at 37°C in an anaerobic chamber containing 80% N_2, 10% H_2, and 10% CO_2. Before initiating bacterial growth, fresh growth medium was incubated in an anaerobic chamber for 3 days to remove any residual oxygen.

Light Apparatus

The apparatuses used in this study were non-laser light sources: a high power blue-colored LED (a device modified from a Flashmax 2^{TM}, CMS Dental, Denmark; wavelength 425–500 nm, power density 4 W/cm^2, which is a light source for composite resin polymerization), and a red LED [Fotosan^{TM}, CMS Dental, Denmark; wavelength 625–635 nm, power density 2 W/cm^2; a light source used in the commercially available Fotosan^{TM} kit for antimicrobial photodynamic therapy (a-PDT)]. The apparatuses were fixed onto a stand within an anaerobic chamber (Forma Scientific 1024, Ontario, Canada), so that the light-emitting end (6.8 mm in diameter) of the LED apparatus was positioned to match the top entrance of a well (6.8 mm in diameter) in a 96-well titer plate during irradiation. The LED irradiation was performed with the light-emitting end in direct contact with the entrance of the well. The distance between the top surface of the bacterial suspension and the light-emitting end was 4.5 mm and the depth of the mixed bacterial suspension was 7.5 mm. The actual energy level on the surface of the mixed solution was approximately 480 and 240 mW for the blue and red LED, respectively (Power meter: NOVA II, Optical Metrology Ltd., Jerusalem, Israel; detection head: PD 300, 3 W) and the power density was calculated to be approximately 1.5 and 0.75 W/cm^2, respectively.

LED Irradiation

P. gingivalis was grown to late-log phase and then adjusted the $OD_{600\ nm}$ was adjusted to 1.0 with new broth. The bacterial suspension (240 μl) was put into a well of the 96-well titer plate and irradiated with blue LED for 90 seconds (15 seconds intervals between 15 seconds irradiation cycles to avoid heat generation, approximately equal to 135 J/cm^2 of total energy), or red LED for 180 seconds (15 seconds intervals between 30 seconds irradiation cycles, approximately equal to 135 J/cm^2 of total energy) anaerobically. These irradiation parameters produced a temperature elevation of <2°C. As a control, bacteria were exposed to no LED irradiation. The irradiated samples were diluted 10 times with new broth and then were incubated at 37°C in an anaerobic chamber.

Preparation of Total RNA

The cell suspensions were mixed with an RNAprotect^{TM} Bacteria Reagent (Qiagen, Tokyo, Japan) to stabilize the bacterial RNAs. Total RNAs were prepared with the Trizol Reagent (Invitrogen, Carlsbad, CA) and a PureLink^{TM} RNA Micro Kit (Invitrogen) according to the manufacturer's instructions. Bacterial disruptions were performed using a FastPrep FP120 Instrument (Qbiogene, Carlsbad, CA) as described previously [25]. The RNA quality was examined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with a RNA 6000 Nano LabChip Kit (Agilent).

RT-PCR

Reverse transcription was performed with random hexamers (Invitrogen) and Superscript II reverse transcriptase (Invitrogen). Primers were designed from the NCBI database entry for *P. gingivalis* strain ATCC33277 (NC_010729) using the Primer3 software program (http://frodo.wi.mit.edu/) and are shown in Table 1.

End-point PCR was carried out by using Z-Taq (Takara, Shiga, Japan) with a thermal cycler (Dice® TP600, Takara) as follows: 94°C for 30 seconds, followed by 30 cycles of 94°C for 5 seconds, 55°C for 5 seconds, and 72°C for 10 seconds. Quantitative real-time PCR (qRT-PCR) was performed using a real-time DNA thermal analyzer (Rotor-Gene^{TM} 6000, Corbett Life Science, Sidney,

TABLE 1. PCR Primers Used in This Study

Gene ID[a]	Gene name	Direction	Primer sequence (5′–3′)	Product size (bp)	Description
PGN_0001	*dnaA*	F	TTTGGAGGGCAATTTCGTAG	124	Chromosome replication initiator
		R	TGTCACCGTACCGGGATATT		
PGN_0034		F	GCAATCGTTTAGCCAAGCTC	132	DNA polymerase III α subunit
		R	GGGTATCGCGCATTACCTTA		
PGN_0041	*htpG*	F	AATGGAAAGACGGCAAGATG	91	Heat shock protein 90
		R	TTGAGGTCAGCAGGCTTTTT		
PGN_0043	*ftsH*	F	GTAGGAGCCTCTCGTGTTCG	132	Cell division protein
		R	CTCATCATTGCCGGAGAAAT		
PGN_0221		F	CTCCGTGGCTACGAAGAAAG	131	DNA topoisomerase III
		R	CATACAGCGACTGGATGGTG		
PGN_0251	*Lon*	F	CCCAAGTGACCGATGAAGTT	146	Malonyl CoA-acyl carrier protein transacylase
		R	ACCGACAGCGAGCTTAAGAG		
PGN_0348	*uspA*	F	GAACTGGGCTTCAACTTTGC	125	Universal stress protein family
		R	TCATTGCTGAGCTGGAACAC		
PGN_0411	*terC*	F	GAGCGGAGGATGGTTGAATA	141	Thiol–disulfide interchange protein
		R	ACAATCCAGAGAGCGAGGAA		
PGN_0582		F	CTACCATCGACAAGCTGCAA	102	DNA topoisomerase I
		R	GGTCATACAAAAGCGGTGGT		
PGN_0631	*ftsZ*	F	TACCACCGATCCGGAGTTAG	111	Cell division protein
		R	ACATTGCCAAGGTTGTCTCC		
PGN_1057	*rccA*	F	GAATGGCCACGGAGAAGATA	137	DNA repair
		R	GTAACCGCCTACACCGAGAG		
PGN_1208	*clpB*	F	AGAAACTGCCCCATGTATCG	129	ClpB protein
		R	CTAGCACGATGTGCTCCAAA		
PGN_1378		F	TGCCGATATGGTATGCT7CA	138	Replicative DNA holicase
		R	CGCAAACGTACATCATCCAC		
PGN_1388		F	GCTTCCGGTACCATTAAGCA	146	DNA ligase
		R	ACTTTGAAACCCCACTGACG		
PGN_1451	*groES*	F	ATTCGGCCAAAGAGAAACCT	95	Chaperonin GroES
		R	TACGGTGTCTCCTGCTTTGA		
PGN_1452	*groEL*	F	TAGAATTGGAGTGCCCGTTC	153	Chaperonin GroEL
		R	CTCCTGCCGTAACGTTCTTC		
PGN_1525	*sulA*	F	CGCTGGGAGATCGTTACATT	101	Capsular polysaccharide biosynthesis protein
		R	GGGGGAAAGACCTTTGAATC		
PGN_1751		F	TGTGCAGAAGTTCCAACTCG	136	DNA primase
		R	TAAGGCCTTGCTGTCTTCGT		
PGN_1771		F	GAGACCGACTCGAAAGATGC	137	DNA polymerase I
		R	AGCGGACGCAAGAGATCTAA		
PGN_1786		F	GAGCCAAGCGCGTATCTATC	118	DNA polymerase III β chain
		R	TCTTCAGCAGCCACAGAGAA		

F, forward; R, reverse.
[a]Locus number from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NC_010729.1).

Australia) with SYBR Premix Ex Taq™ (Takara) as follows: 95°C for 5 seconds, followed by 40 cycles of 95°C for 10 seconds, 57°C for 15 seconds, and 72°C for 20 seconds. All data were analyzed based on the comparative CT method [26]. The resulting relative values represented the relative expression level of a given gene compared with the level in the non-irradiated control at a corresponding incubation time point.

Statistical Analysis

Data are presented as the means ± SD of triplicate experiments. A statistical analysis was performed using Student's *t*-test. Values of $P < 0.05$ were considered to be significant.

RESULTS

Effect of the LED Irradiation on the Growth of *P. gingivalis*

The growth curves for *P. gingivalis* after LED irradiation (135 J/cm^2) are shown in Figure 1 compared with the non-irradiated control, the blue LED significantly delayed the growth of *P. gingivalis* ($P < 0.05$), whereas the red LED did not.

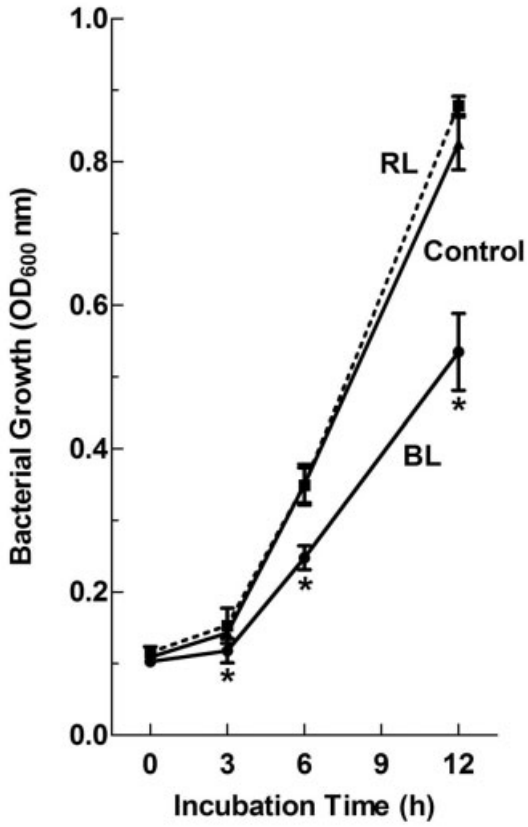

Fig. 1. Growth curves of *P. gingivalis* after LED irradiation. The optical density ($OD_{600\ nm}$) was measured at 3, 6, 9, and 12 hours after irradiation (135 J/cm^2) with the blue LED (BL, circle, solid line) or red LED (RL, square, dashed line). Control bacteria without any irradiation were also examined (triangle, solid line). The results are presented as the means ± SD ($n = 3$). The error bars represent the confidence interval at 95%. Significant differences ($P < 0.05$) are labeled with asterisks.

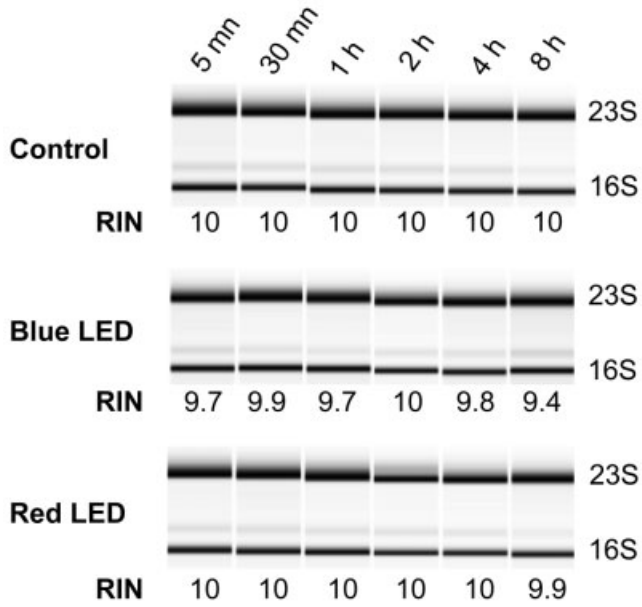

Fig. 2. Electrophoretic analysis of the total RNA extracts. Purified RNA samples of *P. gingivalis* obtained during the growth were examined using a 2100 electrophoresis Bioanalyzer (Agilent Technologies). The major ribosomal RNA bands are indicated (23S and 16S rRNA). The RNA integrity number (RIN) indicates the extent of RNA degradation. The values (1–10) are shown below the image.

Investigation of the Bacterial Viability After Irradiation

The degradation of total RNAs was examined based on the RNA integrity number (RIN) using irradiated and non-irradiated samples collected 5, 30 minutes, 1, 2, 4, and 8 hours after culture with/without LED irradiation (Fig. 2). The RIN numbers are valued from 1 to 10, with a value of 10 indicating a perfect RNA sample without any degradation products, whereas a value of one indicates a completely degraded sample [27]. In this study, all samples showed RIN values from 9.4 to 10.0, without any remarkable differences between the samples exposed to the blue LED and red LED, thus indicating that there was no significant degradation in any of the samples.

In addition, RT-PCR analyses of stress-related genes were carried out (Fig. 3). In 15 minutes post-irradiation sample subjected to irradiation with blue LED, the *clpB* (PGN_1208) expression increased remarkably. However, there were no differences in the expression levels of *htpG* (PGN_0041), *uspA* (PGN_0348), *groES* (PGN_1451), and *groEL* (PGN_1452) compared to the non-irradiated control.

Analysis of the Expression of Genes Related to DNA Replication and Cell Division

We next hypothesized that the growth inhibitory effect of blue LED may be due to the downregulation of the expression of DNA replication- and cell division-related genes. Thus, we examined the gene expression levels using post-irradiation samples (5, 15 minutes, 1, 2, and 4 hours) and compared them with the non-irradiated samples by qRT-PCR (Fig. 4). As controls, both red LED-irradiated and non-irradiated samples were also examined. Red LED radiation did not show any significant effects compared with the non-irradiated control from 5 minutes to 4 hours post-irradiation. In contrast, the expression levels in the blue LED irradiation group were inhibited maximally at 5 minutes post-irradiation; the most remarkable decrease was shown in the gene encoding DNA ligase (PGN_1388), which was decreased by 13.7-fold compared to the non-irradiated samples, followed by the gene encoding the thiol–disulfide interchange protein (*terC*; PGN_0411, 6.3-fold), DNA topoisomerase I (PGN_0582, 5.6-fold), chromosome replication initiator (*dnaA*; PGN_0001, 4.4-fold), cell division

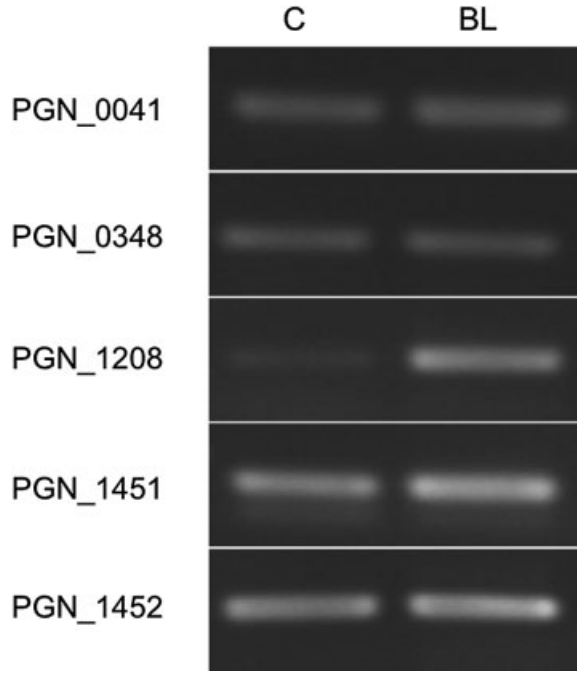

Fig. 3. The expression profiles of stress-related genes. RNA was extracted from *P. gingivalis* 15 minutes after irradiation (135 J/cm^2). The gene expression was measured by end-point RCR (30 cycles). Non-irradiated samples were used as a control (C). BL: blue LED.

protein (*ftsH*; PGN_0043, 4.4-fold), DNA helicase (PGN_1378, 4.0-fold), and DNA polymerase I (PGN_1771, 3.2-fold)/III (PGN_0034, 3.7-fold). The differences in the Blue LED and non-irradiation groups gradually decreased, and they no longer exhibited any differences after 1–2 hours.

DISCUSSION

In our study, all processes from LED irradiation to RNA stabilization were performed under anaerobic conditions in order to avoid the influence of oxygen. The red LED irradiation (135 J/cm^2) did not lead to any differences in the bacterial cells compared to the non-irradiated control in terms of their growth. In contrast, blue LED irradiation (135 J/cm^2) delayed the growth of *P. gingivalis*, which was in agreement with the results of previous studies [9,20–23,28,29]. However, the molecular mechanisms underlying the effects of blue light/LED exposure on bacterial growth were unclear with regard to whether blue light/LED light actually kills bacteria or only inhibits their growth.

The ability of bacteria to grow in selective media and to form colonies on nutrient agar plates is routinely used as a retrospective criterion for the detection of living bacteria [30]. The ability of bacteria to proliferate is generally examined by measuring the colony forming units (CFUs) on an agar plate or by assessing the absorbance of the growth medium. Under some circumstances, the number of viable microorganisms may be underrepresented by such methods, because there may be sublethally damaged organisms [31] or fastidious uncultivable bacteria [25]. A number of bacterial species have been observed to undergo metabolic changes leading to the production of cells that can no longer actively form colonies on solid media, but that retain other indicators of cell viability, such as active membrane potential, maintenance of cellular integrity and the capacity for metabolic activity [26–29], thus indicating that it may be difficult to accurately assess the viable bacteria by measuring the CFUs or the absorbance.

Several molecular candidates have been reported for the more accurate assessment of the viability of bacteria [32]. The presence of intact DNA was initially used as an indicator of cell viability with the assumption that the DNA would be degraded in a dead cell more rapidly than other cellular components [33]. However, the precise correlation of cell viability with the detection of DNA was shown to be poor, with DNA persisting in actively killed cells for a significant period of time [34]. DNA was also demonstrated to persist in a PCR-detectable form in a culture-negative environment and in clinical samples even after the chromosomal DNA has begun to disintegrate [35]. In contrast, RNA is known to be a highly labile molecule with a very short half-life (seconds), and therefore it should provide a more closely correlated indication of the viability status than DNA-based methods [32]. However, in several studies, it has been shown that both DNA and RNA may persist in a detectable form for several hours after cell death, confirming that there may also be a poor correlation between mRNA detection and cell viability [30,36,37]. On the other hand, recent technology has now made it possible to assess the extent of RNA degradation using the RIN value [27]. Thus, in the present study, we isolated total RNAs from the non-irradiated and post-irradiated samples (until 8 hours). No significant RNA degradation was observed in any of the red or blue LED-irradiated samples, suggesting that LED may not induce the death of *P. gingivalis*.

A diverse range of stresses, such as heat shock, salt stress, ethanol, starvation for oxygen or nutrients, etc. induce the same set of proteins, called general stress proteins. These are proposed to provide general and non-specific protection of cells under these adverse conditions. The bacterial stress genes can be used as markers to monitor the fitness of industrial bacterial hosts during fermentation processes [38]. More than 200 genes, including these stress genes, are induced at least 3.0-fold within the first minute after exposure to heat stress in *Bacillus subtilis* [39]. In addition, the expression of stress-related genes has been reported to be an indicator for the viability of bacteria [40]. Microbiological, cytological, and molecular analyses of the physiological response to lethal heat stress under accurately defined conditions can be achieved through systematic sampling of bacteria from a single culture exposed to gradually increasing temperatures. Essential genes encoding chaperons of the protein folding mechanism are not expressed by dead bacteria, but instead by living bacteria. These can serve

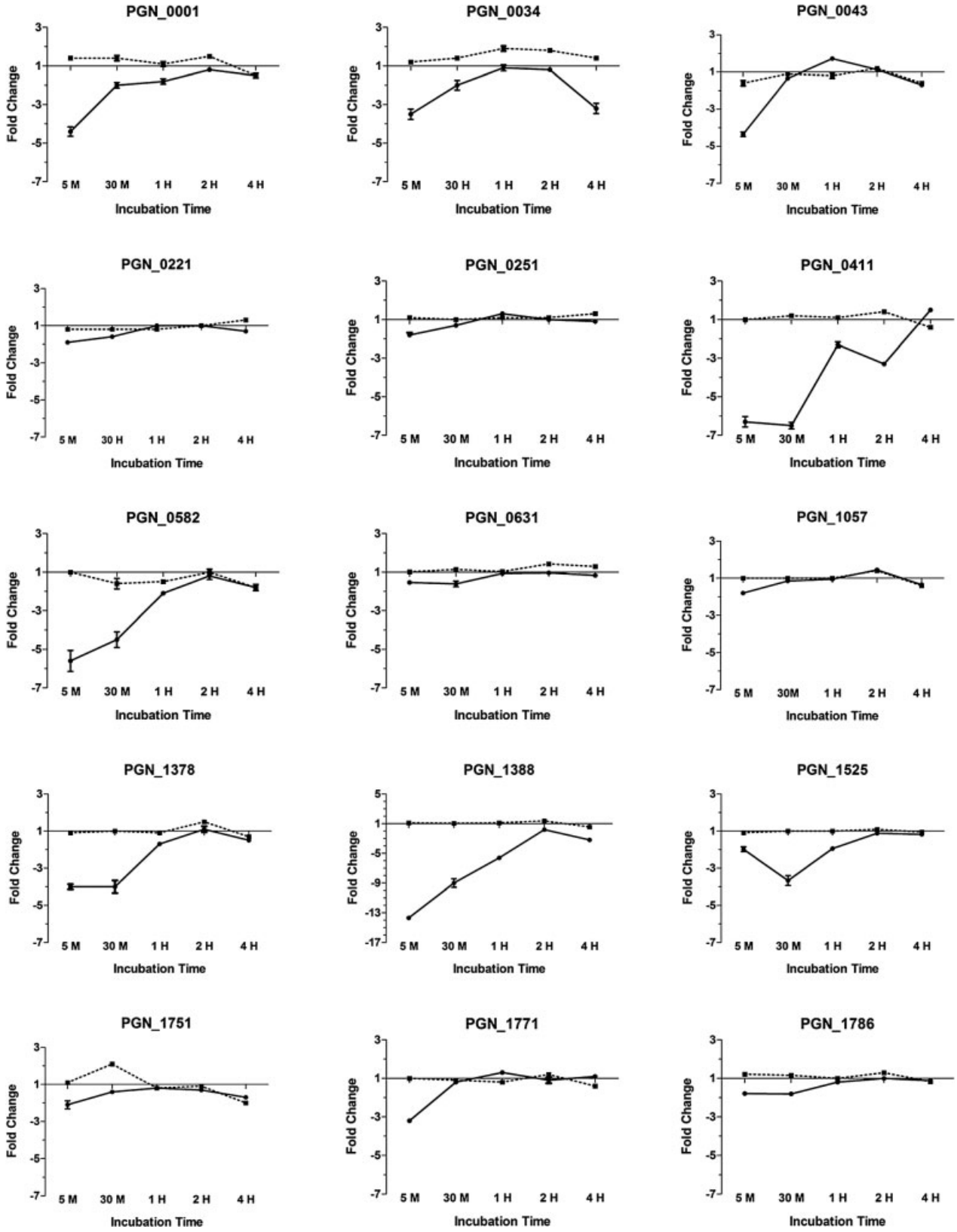

Fig. 4. Gene expression profiling after irradiation with blue or red LED. Total RNA was extracted from *P. gingivalis* after incubation for different lengths of time after irradiation (135 J/cm^2) with the blue LED (circle, solid line) or red LED (square, dashed line). The gene expression was measured by qRT-PCR of the irradiated samples. Non-irradiated samples at each time points were used as a control for normalization. The results are presented as the means $\pm$ SD ($n = 3$) of the fold change in gene expression at each of the time points calculated based on the comparative CT method. M: minute; H: hour.

as molecular biomarkers for bacterial cell viability. Therefore, the expression levels of stress-related genes, such as *clpB*, *htpG*, *uspA*, *groES*, and *groEL* were also investigated in this study. Their gene expression levels remained constant or increased 15 minutes after the blue LED irradiation compared with before irradiation, suggesting that there was no bacterial cell death in the blue LED-irradiated bacteria. Together, our results regarding the RNA degradation and the gene expression levels of stress proteins indicate that the growth inhibition of *P. gingivalis* by the blue LED may be induced not by a bactericidal effect, but instead due to a bacteriostatic effect.

In order to elucidate the mechanism underlying the bacterial growth inhibition induced by blue LED irradiation, we focused on the genes encoding proteins related to chromosome replication and cell division. The blue LED strongly inhibited the expression of genes encoding DNA ligase, DNA topoisomerase I, DnaA, FtsH, DNA helicase, and DNA polymerase at 5 minutes post-irradiation and their transcript does not persist for more than 1–2 hours. There have been many reports about the relationship between bacterial growth and the genes examined in this study. For example, the chromosome replication initiator, *dnaA*, plays a key role in the initiation of chromosomal DNA replication. A *dnaA* mutant led to the inhibition of cell division and proliferation [41]. An *E. coli* mutant of the DNA polymerase III α subunit has been shown to increase the resistance to replication-pausing on the template DNA, a high capacity to promote strand-displacement DNA synthesis, and a reduced rate of DNA chain elongation [42]. In *Streptomyces coelicolor*, a polymerase I mutant grew more slowly than the wild type strain, with smaller colony sizes [43], thus suggesting that this probably reflects a retardation in the joining of Okazaki fragments [44,45]. FtsH, a cell division-related protein, has been reported to play a very important role in bacterial growth [46]. A *ftsH* mutant showed relatively slower growth compared to the wild type strain [47] and failed to undergo the typical growth phase-dependent morphological adaptation observed for wild type *Caulobacter crescentus* [48]. These previous reports may support our results showing the inhibition of *P. gingivalis* growth by blue LED irradiation.

In the present study, the gene encoding DNA ligase (PGN_1388 in a strain ATCC33277) showed the most significantly decreased expression (13.7-fold) in comparison with the non-irradiation control at 5 minutes post-irradiation with blue LED. The predicted amino acid sequence has high homology with other *P. gingivalis* strains, such as W83 (99%, DNA ligase, NAD-dependent acetyltransferase, GNAT family) and TDC60 (99%, NAD-dependent DNA ligase acetyltransferase). The addition of a DNA ligase inhibitor to the growth medium inhibited the DNA replication and promoted the accumulation of polynucleotide fragments of a size consistent with Okazaki fragments [49]. Recently, the NAD-dependent DNA ligases have been demonstrated to represent a potentially selective target for the discovery and development of novel broad-spectrum antibacterial agents because it is indispensable for DNA replication, conserved among bacterial pathogens, and distinctly different from the eukaryotic DNA ligases [49–53]. Here, the blue LED did not have any killing effect, even though the gene expression was downregulated just after irradiation, thus suggesting that the blue LED irradiation energy may be insufficient for permanent inhibition of the DNA ligase gene expression. Regarding the use of blue LED for clinical applications, unfortunately, the effect of blue LED is not bactericidal but bacteriostatic. However, the growth inhibition effect of blue LED is advantageous. In the clinical application of blue LED, repeated irradiation within a short time period may therefore be effective for bacterial reduction, and also with the application of a-PDT system, the synergic effect for such bacterial killing might also be expected using blue LED in combination with dye agents.

CONCLUSIONS

Blue LED irradiation inhibited the growth of *P. gingivalis* by suppressing the expression of the genes associated with chromosomal DNA replication and cell division at the transcriptional level. However, the signaling pathway(s) activated by the blue LED irradiation with regard to this suppression of gene expression are still unknown. Future studies including signal pathway analyses will be required to clarify the mechanism responsible for the bacterial growth inhibition induced by blue LED. In addition, all experiments were conducted using one laboratory strain in this study. Clinical isolates should also be investigated to confirm the effect of blue LED light.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Global Center of Excellence Program for International Research Center for Molecular Science in Tooth and Bone Diseases at Tokyo Medical and Dental University from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and by a Grant-in-Aid for Scientific Research (C) (22592308) to A.A. and (B) (21390497) to Y.A. from Japan Society for the Promotion of Science (JSPS).

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Journal of
PERIODONTAL RESEARCH

J Periodontal Res 2013
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Published by Blackwell Publishing Ltd.
JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12055

Antimicrobial effect of photodynamic therapy using high-power blue light-emitting diode and red-dye agent on *Porphyromonas gingivalis*

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Chui C, Aoki A, Takeuchi Y, Sasaki Y, Hiratsuka K, Abiko Y, Izumi Y. Antimicrobial effect of photodynamic therapy using high-power blue light-emitting diode and red-dye agent on Porphyromonas gingivalis. J Periodont Res 2013; doi: 10.1111/jre.12055. © 2013 John Wiley & Sons A/S. Published by Blackwell Publishing Ltd.

Background and Objective: Antimicrobial photodynamic therapy (a-PDT) using a combination of red-colored laser/light-emitting diode (LED) and blue dye has been employed for periodontal therapy and the antimicrobial effect seems promising. Blue light, which has favorable wavelength properties, would be more effective as a light source for a-PDT because blue light itself possesses an antimicrobial effect. This study aimed to investigate the effect of a-PDT using a novel combination of high-power blue LED and red-dye agent on *Porphyromonas gingivalis in vitro*.

Material and Methods: Porphyromonas gingivalis ATCC 33277 suspension was irradiated with blue LED (BL) (425–470 nm) or red LED (RL) (625–635 nm) at 30–90 J/cm^2, or was mixed with erythrosine (ER), phloxine B (PB) or rose bengal (RB) with or without BL irradiation (30 J/cm^2). RL (30 J/cm^2) in combination with toluidine blue was employed as positive control. All the suspensions of *P. gingivalis* were serially diluted, plated and incubated anaerobically, and the numbers of colony-forming units (CFUs) were counted on day 7.

Results: BL irradiation at 60 and 90 J/cm^2 demonstrated a significant reduction in the numbers of CFUs. ER, PB and RB solutions at 160 μg/mL showed almost no or only a minimal reduction in the numbers of CFUs. BL at 30 J/cm^2 combined with ER, PB or RB at 160 μg/mL resulted in a log reduction of 0.9, 1.0 and 7.1, respectively, in the numbers of CFUs; 30 J/cm^2 BL with RB at 1.6, 16 and 160 μg/mL demonstrated a log reduction of 6.3, 8.0 and 5.5, respectively; and a log reduction of 5.2 was obtained after 30 J/cm^2 RL with 16 μg/mL TB.

Conclusion: Within the limits of this study, BL was found to have an antimicrobial/growth-inhibiting effect on *P. gingivalis*, and a-PDT using a combination of BL and RB shows promise as a new technical modality for bacterial elimination in periodontal therapy.

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Key words: antimicrobial photodynamic therapy; blue light-emitting diode; *Porphyromonas gingivalis*; rose bengal

Accepted for publication January 08, 2013

Periodontal diseases derive from inflammation of the tooth-supporting periodontal tissues in response to chronic infections caused by periodontopathic bacteria. The aim of periodontal treatment is to arrest infection and inflammation of periodontal tissues. However, it has been reported that conventional treatment approaches, such as mechanical therapy (1) and chemotherapy (2), do not necessarily eradicate bacteria completely. Chemotherapy using antibiotics has many disadvantages: it may cause side effects such as allergy; it is difficult to maintain the therapeutic concentration in periodontal pockets; and there is a high possibility that microorganisms develop resistance with repeated usage (3–6). *Porphyromonas gingivalis* is a black-pigmented, gram-negative anaerobic bacterium, which is thought to be an important etiological agent in adult periodontal disease (7,8). It possesses many virulence factors (such as fimbriae, protease, hemagglutinins and capsular polysaccharide) that allow it to cause disease (7). This bacterium is known to mediate the co-aggregation of various oral gram-positive and gram-negative bacteria. In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilms containing multiple species that colonize specific surfaces and result in interactive nutritional chains (9).

Recently, light energy, including various lasers, has been applied in the field of periodontal therapy. Laser is an artificial light that has certain unique properties, namely high monochromaticity, coherence and directionality, compared with ordinary sources of light, although both are electromagnetic radiations. High-power lasers, which are strongly bactericidal, have been successfully employed as an adjunct tool for the past two decades (10–12). However, they may also cause excessive thermal denaturation, in the form of carbonization, coagulation and melting, on the tooth structures and on periodontal and pulpal tissues during irradiation (10–13).

Quite recently, a new treatment approach using light energy – antimicrobial photodynamic therapy (a-PDT) – based on a photochemical reaction was introduced for effective elimination of periodontal infection. a-PDT is a noninvasive treatment procedure that uses low-level light energy to activate a photosensitizing agent in the presence of oxygen, leading to the production of reactive oxygen species that cause irreparable biological damage to bacterial or target cells (14–17). a-PDT has three components – visible harmless light, a nontoxic photosensitizer and oxygen – and does not produce any resistance in microorganisms (18).

In the field of periodontal therapy, the antimicrobial effect of PDT has been studied and clinically applied using the combination of a red-colored diode laser light and a blue dye such as toluidine blue (TB) or methylene blue (17). On the other hand, considering the wavelength property, blue light would be more promising as the light source in a-PDT than the red light because the blue light itself exhibits an antimicrobial effect (9,19–22). Also, compared with the diode laser, the light-emitting diode (LED) would be more advantageous as a light source in clinical application in terms of the lower device cost.

The aim of this *in-vitro* study was to investigate the antimicrobial effect of a high-power blue-colored LED and that of red-dye agents, separately and in combination, as a novel a-PDT therapy on one of the major periodontopathic bacteria, *P. gingivalis* ATCC 33277.

Material and methods

Light apparatus

The following devices were used in this study as nonlaser light sources: a high-power blue LED (BL) (a device modified from Flashmax2™; CMS Dental, Copenhagen, Denmark) (wavelength 425–500 nm, approximate power density 4 W/cm^2) and a high-power red LED (RL) (Fotosan™; CMS Dental) (wavelength 625–635 nm, power density 2 W/cm^2, a light source used in the commercially available Fotosan kit for a-PDT). The apparatus was fixed onto a stand so that the light-emitting end (6.8 mm in diameter) of the LED apparatus was positioned to correspond with the opening of a well (6.8 mm in diameter) in a 96-well plate during irradiation.

Preparation of bacterial suspension

P. gingivalis ATCC 33277 was maintained on brucella agar plates (supplemented with 50 mL/L of horse blood, 5 mg/L of hemin and 50 μg/L of vitamin K_1) at 37°C under anaerobic conditions. A loopful of the bacterium was inoculated into 9 mL of brain–heart infusion medium (supplemented with 5 mg/L of hemin and 50 μg/L of vitamin K_1) and cultured anaerobically for 24 h at 37°C. Immediately before the experiment, the bacterial suspension was diluted and the concentration was adjusted to 1×10^8 cells/mL using a counting chamber.

Preparation of dye and other solutions

Three different red-dye agents – erythrosine (ER), phloxine B (PB) and rose bengal (RB) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) – were employed. Solutions of all three dyes were freshly prepared before each experiment using sterile physiologic saline. Also, two different commercially available dental plaque-disclosing agents were used: Red Dye A [Dent liquid plaque tester® (previous version, not on sale at present); Lion Dental Products Co., Ltd., Tokyo, Japan] and Red Dye B (Butler GUM Red-Cote®, Dental disclosing solution; Sunstar Americas, Inc., Chicago, IL, USA). Both agents contained the same red-colored dye, PB, at undisclosed concentrations. Twenty per cent chlorhexidine gluconate (CHG) solution (Wako Pure Chemical Industries, Osaka, Japan) was

diluted with sterile distilled water to obtain solutions of 0.72% and 1.2% so that the final concentration after mixing with the bacterial suspension was 0.12% and 0.2%, respectively (which are the standard concentrations used in mouthwash). TB powder (Sigma-Aldrich, St Louis, MO, USA) was dissolved in saline to obtain a concentration of 0.1 mg/mL, which is equivalent to the concentration of TB solution commercially available in the Fotosan a-PDT kit.

Experiment 1: antimicrobial effect of high-power BL and RL on *P. gingivalis*

Eight groups were employed in this experiment: one nonirradiated control group; three BL irradiation groups and two RL irradiation groups with different time-periods of irradiation; one positive control group of conventional a-PDT using TB + RL; and a control group of TB only. For each group, a 50-μL aliquot of the bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate and mixed with either 10 μL of reduced dilution buffer (23) for the nonirradiated control group and the BL and RL irradiation groups or 10 μL of TB solution (0.1 mg/mL, the final concentration in the mixed solution 16 μg/mL) for TB + RL and TB only groups. In the TB + RL and TB only groups, the mixed solution was allowed to stand for 1 min to permit the uptake of dye by bacteria before irradiation or culture. In the LED irradiation groups, the mixed solution was irradiated with BL for 30, 60 and 90 s (total power density approximately 30, 60 and 90 J/cm^2, respectively) or with RL for 60 and 120 s (total power density approximately 30 and 60 J/cm^2, respectively). The TB + RL group was exposed to 60 s of RL irradiation (30 J/cm^2). Each bacterial suspension was individually exposed to LED irradiation after preparation of the suspension in each well. The LED irradiation was performed with the light-emitting end in direct contact with the entrance of the well. The distance between the top surface of the mixed bacterial suspension and the light-emitting end was 7.5 mm and the depth of the mixed bacterial suspension was 2.5 mm. The actual energy level at the surface of the mixed solution was approximately 365 mW and 185 mW for BL and RL, respectively (power meter: NOVA II; Optical Metrology Ltd., Jerusalem, Israel; detection head: PD 300, 3W) and the power density was calculated to be approximately 1.0 and 0.5 W/cm^2, respectively.

After treatment, 20 μL of sample from each group was serially diluted by 10^3–to 10^5– fold using dilution buffer and then 10 μL of diluted samples were plated, in triplicate, on brucella agar plates. The experiment was performed under aerobic conditions and all procedures were completed within 30 min. During the experiment, a tube containing the original bacterial suspension was kept in a glass container with crushed ice. After anaerobic incubation for 7 d at 37°C, the numbers of colony-forming units (CFUs) were determined. The experiment was repeated five times, independently.

Experiment 2: antimicrobial effect of red-dye agents

The antimicrobial effect of red-dye agents on *P. gingivalis* was investigated and the optimal concentration of the dye for a-PDT in combination with BL was estimated. Twelve groups were employed in this experiment: one untreated control group; two groups of commercially available dental plaque-disclosing agents; and nine groups of the experimental red-dye agents ER, PB or RB, at three different concentrations each.

For each group, a 50-μL aliquot of fresh *P. gingivalis* suspension was placed in a well of a sterile 96-well flat-bottom plate and mixed with 10 μL of dilution buffer (the untreated control) and 10 μL of Red Dye A (Dental liquid plaque tester®) or 10 μL of Red Dye B (Butler GUM Red-Cote®) (both contain PB) at the original concentration for plaque-disclosing agent groups, or with 10 μL of the experimental red dyes RB, ER or PB (1, 10 and 50 mg/mL of each dye solution; the final concentration: 0.16, 1.6 and 8.3 mg/mL, respectively). The mixed solutions were allowed to stand for 1 min. Finally, these solutions were separately serially diluted with dilution buffer, plated onto brucella agar plates in triplicate and incubated anaerobically. The numbers of CFUs were determined. The experiment was repeated five times independently.

Experiment 3: antimicrobial effect of BL in combination with ER, PB and RB

The antimicrobial effect of BL in combination with red-dye agents was investigated. ER, PB and RB solutions were prepared at 1 mg/mL in normal saline. Eight groups were employed in this experiment: one untreated control group; six groups of ER, PB or RB with/without BL irradiation; and one group of BL only.

In each group, 50 μL of bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate, mixed with 10 μL of dilution buffer for control and BL-only groups or with 10 μL of each dye solution (1 mg/mL; the final concentration was 0.16 mg/mL) for ER, PB and RB groups, and allowed to stand for 1 min. One of the two wells treated with each dye solution or dilution buffer was irradiated (30 J/cm^2) with BL for 30 s. Finally, each group was serially diluted, plated in triplicate onto brucella agar plates and incubated anaerobically.

Experiment 4: antimicrobial effect of BL in combination with graded concentrations of RB

The antimicrobial effect of PDT with different concentrations of RB solutions was examined. Thirteen groups were employed in this experiment: one untreated control group; eight groups of RB at four different concentrations with/without BL irradiation; one group of BL irradiation; two groups of CHG (positive controls); and another positive control of conventional a-PDT with TB + RL.

In each group, 50 μL of bacterial suspension was placed in a well of a sterile 96-well flat-bottom plate and

then mixed with dilution buffer (control group), with each concentration of RB solutions (0.001, 0.01, 0.1 and 1 mg/mL; the final concentrations were 0.16, 1.6, 16 and 160 μg/mL, respectively) for the RB with/without BL groups, with each concentration of CHG (0.72% and 1.2%, the final concentrations were 0.12% and 0.2%) for the CHG groups or with TB solution (0.1 mg/mL, the final concentration was 16 μg/mL) for the TB + RL group and allowed to stand for 1 min. One of the two wells mixed with RB solution at each concentration was exposed to BL for 30 s (30 J/cm^2). One well mixed with TB was irradiated with RL for 60 s (30 J/cm^2). Finally, each group was serially diluted, plated in triplicate onto brucella agar plates and incubated anaerobically.

Experiment 5: temperature measurement of the bacterial suspension during LED irradiation

A thermocouple (Digital Thermometer IT-2000; Iuchi Seieido, Osaka, Japan) was used to measure the degree of temperature elevation of the bacterial suspension during the BL (30–90 J/cm^2) and RL (30–60 J/cm^2) irradiation and a-PDT using 30 J/cm^2 BL with RB (0.001–1 mg/mL). Before and immediately after irradiation, a thermocouple was inserted into the suspension and the temperature at the vertical and horizontal middle of the suspension was measured. Temperature measurement was performed in five independent experiments.

Statistical analysis

For each experiment, data are presented as mean ± standard deviation of five independent experiments. All statistical analyses were performed using JMP® 9.0.3 (released by SAS Institute Inc., Cary, NC, USA). One-way analysis of variance was used for all the group comparisons except for experiment 2 (Table 1). Post-hoc Dunnett's test was used to compare differences between each group and control, except for experiments 2 (Table 2), 4 (Fig. 4), and 5 (Tables 3 and 4) where Tukey's test was performed to compare the differences among all the groups. In experiment 2 (Table 1), two-way analysis of variance was performed followed by Tamhane to compare the factor of dye, factor of concentration and the interaction between the two factors. A p value of < 0.05 was considered significant.

Results

Experiment 1: effect of high-power BL and RL on *P. gingivalis*

BL irradiation at 60 and 90 J/cm^2 showed a similar weak, but statistically significant, antimicrobial effect with a 1.1 and 1.2 log reduction in bacteria compared with the control ($p < 0.05$). There was almost no effect of RL at 30 and 60 J/cm^2. a-PDT with RL (30 J/cm^2) and TB (16 μg/mL) showed a moderate effect, with a 2.9 log reduction in bacteria compared with the control ($p < 0.0001$) (Fig. 1).

Experiment 2: effect of red-dye agents

The antimicrobial effect of red dyes on the viability of the *P. gingivalis* suspension is shown in Tables 1 and 2. Dyes A and B used at the commercially available concentration resulted in 7.0 log ($p < 0.0001$) and 1.7 log ($p < 0.05$) reductions of bacteria, respectively, compared with the control (Table 2). All three experimental red-dye agents showed low to high

Table 1. Effect of red-dye agent

		Bacterial count ($\log_{10}$ CFU/mL)			
		Red-dye agent			Control
Dye concentration (mg/mL)	Final concentration (mg/mL)	ER	PB	RB	Dilution buffer
1	0.16	8.5 ± 0.9	8.7 ± 0.8	7.9 ± 1.4	
10	1.6	8.0 ± 0.8	7.6 ± 0.4	6.0 ± 1.2	8.1 ± 0.3
50	8.3	7.4 ± 0.6	3.8 ± 2.4	1.7 ± 1.7	

Results are given as mean ± standard deviation.
CFU, colony-forming units; ER, erythrosine; PB, phloxine B; RB, rose bengal.
The factor of dye concentration and the interaction between the two factors were statistically significant at $p < 0.0001$.

Table 2. Effect of plaque-disclosing agent

	Bacterial count ($\log_{10}$ CFU/mL)		
	Red-dye agent		Control
Group	Dye A	Dye B	Dilution buffer
	1.1 ± 1.4	6.5 ± 0.3	8.1 ± 0.3
	***	***	
	*		

Results are given as mean ± standard deviation.
Dye A: Dental liquid plaque tester®. Dye B: Butler GUM Red-Cote®.
CFU, colony-forming units
Statistically significant differences among experimental groups ($n = 5$) are shown as *($p < 0.05$) and ***($p < 0.0001$).

Table 3. Temperature elevation after blue and red light-emitting diode (LED) irradiation

	BL			RL	
				TB (−)	TB (+)
Energy density (J/cm^2)	30	60	90	30	30
Temperature rise (°C)	3.8 ± 0.1	6.4 ± 0.7	8.7 ± 0.9	1.9 ± 0.2	7.2 ± 0.2
	***	***	***	***	

Results are given as mean ± standard deviation.
BL, blue LED; RL, red LED; TB, toluidine blue.
Statistically significant differences among experimental groups ($n = 3$) are shown as ***($p < 0.0001$).

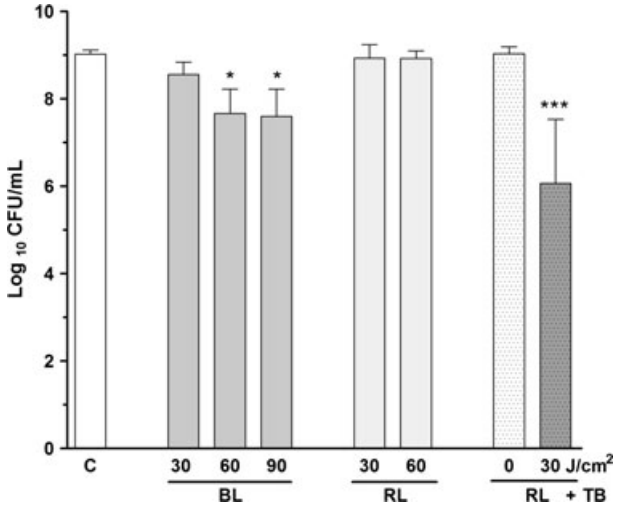

Fig 1. Effect of blue light-emitting diode (BL) and red light-emitting diode (RL) on *Porphyromonas gingivalis* viability. *P. gingivalis* suspension was irradiated with BL at 30–90 J/cm^2 (1 W/cm^2, 30–90 s, respectively) or with RL at 30–60 J/cm^2 (0.5 W/cm^2, 60–180 s, respectively), or was mixed with 16 μg/mL of toluidine blue (TB), with or without RL irradiation at 30 J/cm^2. Nonirradiated bacterial suspension was employed as the control (C). Statistically significant differences between experimental groups ($n = 5$) and the control group ($n = 5$) are shown as *($p < 0.05$) and *** ($p < 0.0001$). CFU, colony-forming units.

reduction in bacteria in a dye concentration-dependent manner. The antimicrobial effect was highest for RB, followed by PB and then ER. At 160 μg/mL, the three dyes showed almost no or only a very minimal antimicrobial effect. The effect of Dye A was higher than that of PB at 8.3 mg/mL (the original dye concentration was 50 mg/mL) and the effect of Dye B was between those of PB at 1.6 and 8.3 mg/mL (original dye concentrations were 10 and 50 mg/mL). The factor of dye concentration and the interaction between the two factors were statistically significant at $p < 0.0001$ (Table 1).

Experiment 3: effect of BL in combination with ER, PB and RB

Figure 2 presents the antimicrobial effect using the combination of 30 J/cm^2 BL and 160 μg/mL of red dye on *P. gingivalis*. Alone, ER, PB, RB and BL resulted in minimal reductions of bacteria, of 0.2, 0.3, 0.6 and 0.5 log, respectively. ER and PB, in combination with BL, also showed similar, minimal, reductions of 0.9 and 1.0 log, respectively. RB + BL demonstrated a significant reduction of 7.1 log compared with the control ($p < 0.0001$) and clearly exhibited an enhanced antimicrobial effect (a-PDT effect).

Experiment 4: effect of BL in combination with graded RB

Figure 3 shows the colors of the RB solution at the original concentrations and after being mixed with the bacterial suspension, and also the conditions before and after irradiation. There were no detectable changes in the condition of the mixed suspensions following irradiation.

In Fig. 4, 30 J/cm^2 BL in combination with 1.6, 16 and 160 μg/mL of RB showed 6.3, 8.0 and 5.5 log reductions, respectively, of bacteria, with a statistically significant difference compared with the control ($p < 0.0001$). RL (30 J/cm^2) combined with 16 μg/mL of TB also demonstrated a significant, 5.2 log, reduction of bacteria ($p < 0.0001$). At 0.12% and 0.2%, CHG showed 2.2 ($p = 0.35$) and a significant 4.3 ($p < 0.001$) log reduction of bacteria, respectively. Among the experimental groups RB + BL and CHG, there was a significant difference between 16 μg/mL of RB + BL and 0.16 μg/mL of RB + BL ($p < 0.001$), between 16 g/mL of RB + BL and 0.12% CHG ($p < 0.0001$) and between 16 μg/mL of RB + BL and 0.2% CHG ($p < 0.05$). A significant difference was also observed between the PDT reactions using 1.6 and 0.16 μg/mL RB ($p < 0.0001$) and between 160 μg/mL of RB + BL and 0.12% CHG ($p < 0.05$). The combination of 16 μg/mL of RB + 30 J/cm^2 BL showed the maximal bacterial reduction among all the groups and a significantly higher reduction than in CHG groups, and there was a tendency for a higher reduction than for the 16 μg/mL TB + 30 J/cm^2 RL group.

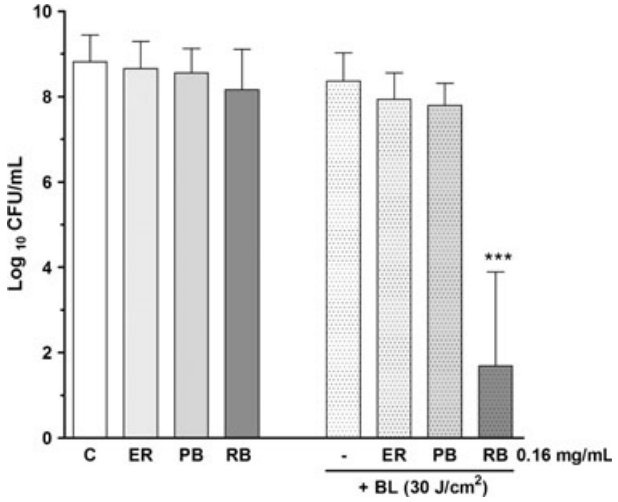

Fig 2. Effect of blue light-emitting diode (BL) in combination with erythrosine (ER), phloxine B (PB) and rose bengal (RB). *Porphyromonas gingivalis* suspension was mixed with ER, PB or RB (1 mg/mL; the final concentration was 0.16 mg/mL) and irradiated with 30 J/cm^2 BL or not irradiated. Bacterial suspension without dye and irradiation was employed as the control (C). Statistically significant differences between experimental groups ($n = 5$) and the control ($n = 5$) are shown as *** ($p < 0.0001$). CFU, colony-forming units.

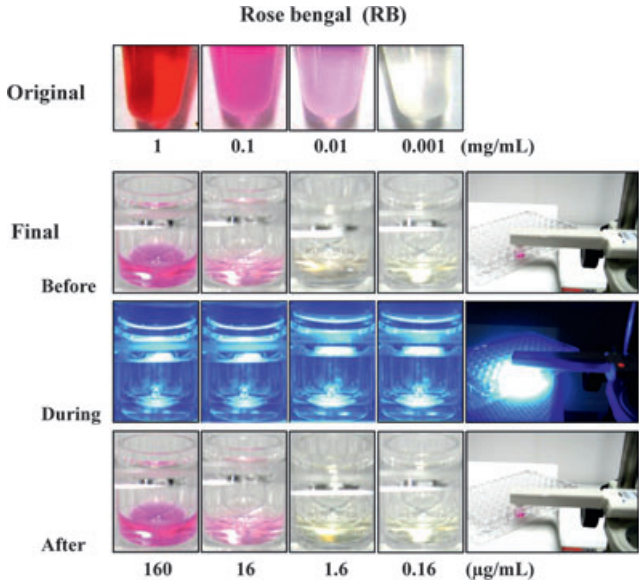

Fig 3. Color of rose bengal at the original concentrations (Original) and at the final concentrations after being mixed with the bacterial suspension (Final); and the conditions of bacterial suspension before, during and after blue light-emitting diode irradiation.

Experiment 5: temperature elevation of the bacterial suspension after LED irradiations

At room temperature (approximately 24°C), BL irradiation increased the temperature significantly in an irradiation time-dependent manner ($p < 0.0001$). There was an average elevation in temperature of 3.8, 6.4 and 8.7°C with 30, 60 and 90 J/cm^2 BL irradiation, respectively. An elevation of 1.9°C was observed with 30 J/cm^2 RL irradiation only and an elevation of 7.2°C was observed with 30 J/cm^2 RL + 16 μg/mL of TB ($p < 0.0001$) compared with 30 J/cm^2 RL irradiation only (Table 3).

In the combination of BL and RB, the temperature of the bacterial suspension was elevated in a dye concentration-dependent manner. Elevations of 3.8, 4.0, 4.3, 4.6 and 7.9°C were observed at 30 J/cm^2 BL with 0, 0.16, 1.6, 16 and 160 μg/mL of RB, respectively. In particular, the temperature showed a marked increase at 160 μg/mL of RB. There was a significant difference in temperature elevations at 1.6, 16 and 160 μg/mL RB compared with BL without RB ($p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively) (Table 4).

Discussion

The present study demonstrates that BL had a moderate antimicrobial effect on *P. gingivalis* ATCC 33277, whilst RL did not affect the bacteria. The difference in the antimicrobial/growth-inhibiting effect between BL and RL may be a result of the difference in the wavelength property of both lights. On BL irradiation, it has been speculated that endogenous porphyrin produced by bacteria is excited, leading to a photodynamic reaction through singlet oxygen production, resulting in an antimicrobial effect (24). In contrast, some *in-vitro* studies reported that red light or near-infrared light exerted no antibacterial effect (25–27).

So far, many studies have reported an antimicrobial effect with blue light. Five minutes of BL delivery (32 J/cm^2) was sufficient to reduce the viability of *Helicobacter pylori* by 99.99% (28). BL irradiation at 58.8 J/cm^2 (9.8 min) reduced the viability of methicillin-resistant *Staphylococcus aureus* US-300 by 92.1% and irradiation at 50.4 J/cm^2 (8.4 min) reduced the viability of *S. aureus* IS-853 by 93.5% (20). BL at a wavelength of 405 nm (15 J/cm^2) achieved reduction rates of 95.1% and nearly 90% for *Pseudomonas aeruginosa* and *S. aureus*, respectively (29). Regarding periodontal pathogens, BL at 94 J/cm^2 (3 min) reduced the survival rates of *P. gingivalis* and *Fusobacterium nucleatum* by 90% and 40%, respectively (25). Moreover, BL irradiation at 4.2 J/cm^2 (1 min) resulted in complete killing of *Prevotella*

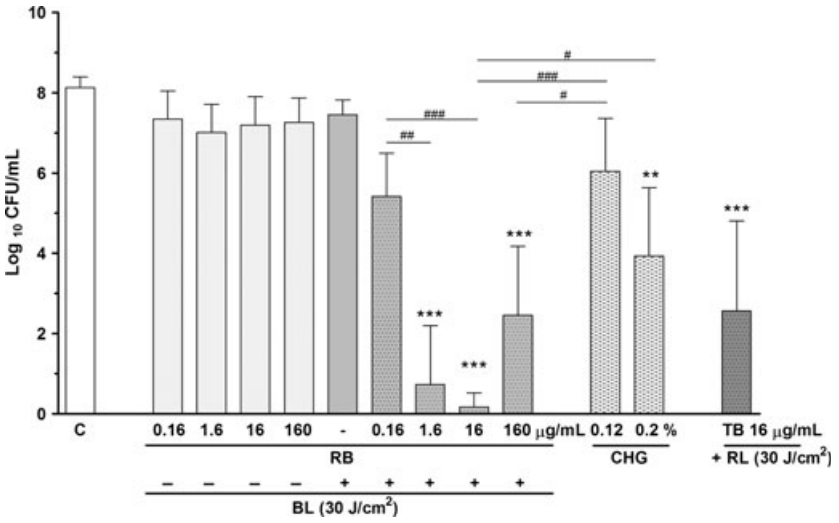

Fig 4. Effect of blue light-emitting diode (BL) in combination with graded rose bengal (RB). Fresh *Porphyromonas gingivalis* suspension was mixed with RB (0.001, 0.01, 0.1 and 1 mg/mL; the final concentrations were 0.16, 1.6, 16 and 160 μg/mL, respectively) and irradiated with 30 J/cm^2 BL or not irradiated. Bacterial suspension incubated with toluidine blue (TB) (0.1 mg/mL, final concentration 16 μg/mL) followed by red light-emitting diode (RL) irradiation at 30 J/cm^2 was employed as the positive control. Bacterial suspension without dye and irradiation was employed as the control (C). Statistically significant differences between experimental groups and the control are shown as **($p < 0.001$) and ***($p < 0.0001$) on each bar. Statistically significant differences among the experimental groups of RB + BL, chlorhexidine gluconate (CHG) and TB + RL are shown as $^{\#}$($p < 0.05$) and $^{\#\#\#}$($p < 0.0001$). CFU, colony-forming units.

intermedia and *Prevotella nigrescens*, and irradiation at 42 J/cm^2 (10 min) reduced *P. gingivalis* by 98.5% (30). Our recent study demonstrated that BL does not kill, but just inhibits the growth of *P. gingivalis* ATCC 33277 (9).

In the present study, we used a high-power BL, expecting a higher antimicrobial effect as well as a shorter irradiation time, which are important in clinical practice. The antimicrobial effect of high-power BL on *P. gingivalis* was detected after short time periods of irradiation of 60 and 90 s, but there was only a 1 log reduction of CFUs. This level of bacterial reduction following treatment with BL alone is not sufficient for clinical application. Therefore, we further investigated the effect of a-PDT using the combination of BL with red-dye agents. Three xanthene dyes – ER, PB and RB – were selected, based on previous studies, as red-dye agents (31,32). Regarding the antimicrobial effects of the dye itself, previously PB has been reported to inhibit methicillin-resistant *S. aureus* (33). RB has also been shown to reduce the viability of all 12 strains of *Enterobacteriaceae* (32) and of planktonic *Aggregatibacter actinomycetemcomitans* (34). Therefore, in the present study, we examined, first of all, the antimicrobial effect of these dyes in order to investigate the toxicity of the red dyes and thereby determine the concentration with the lowest toxicity suitable for a-PDT. RB showed the highest antimicrobial effect on *P. gingivalis* followed by PB and ER. These results coincided with those of quite recent studies by Kato *et al*. (35) on *S. aureus* and by Ishiyama *et al*. (36) on *Streptococcus mutans*. As for the antimicrobial effect of commercially available dental plaque-disclosing agents on *P. gingivalis*, interestingly both Red Dye A and Red Dye B (both contain PB) demonstrated strong (7 log CFU reduction) and slight (1.7 log CFU reduction) effects, respectively. Therefore, the plaque-disclosing procedure in periodontal therapy may have an additional effect on bacterial elimination in the clinic.

The concentration of red dye employed for a-PDT was 160 μg/mL after being mixed with the bacterial suspension in experiment 3 because, at this concentration, the red dyes showed no or only minimal toxicity. In the analysis of a-PDT with BL, among the three dyes in our study, only RB clearly showed an enhanced antimicrobial effect. Recently, Rossoni *et al*. (32) demonstrated that a-PDT with BL and RB was more effective than a-PDT with BL and ER: a-PDT with 50 μmol/L (0.05 mg/mL) of RB and 180 s (200 mW) of irradiation resulted in the reduction of *Enterobacteriaceae* species by 6–7 log whilst a-PDT with 50 μmol/L (0.041 mg/mL) of ER did not show any reduction in the number of CFUs compared with the control (32). Quite recently, Ishiyama *et al*. (36) and Kato *et al*. (35) also showed that among the red dyes employed, RB in combination with green laser/wide-spectrum halogen light exhibited the highest a-PDT activity against *S. mutans* and *S. aureus*, respectively. The result of the present study, indicating the highest effect of a-PDT with RB on *P. gingivalis*, is in agreement with the results of the abovementioned studies.

In a-PDT, matching of the light and the dye is important. However, in the present study, the wavelength of BL (425–500 nm) did not completely match with the maximum absorption wavelength of the red dyes employed: PB (538 nm), ER (526 nm) and RB (549 nm) (35). This discrepancy between the emission wavelength of BL and the absorption wavelengths of red dyes might have influenced the results of the a-PDT effect. However, in previous studies, Ishiyama *et al*. (36) used 532-nm laser light, which matches well with the absorption wavelengths of the three dyes, and they reported that only RB exerted an a-PDT effect. Also, in the study of Kato *et al*. (35), halogen light, which produces a continuous spectrum of light from near-ultraviolet to deep into the infrared region, was employed and the result also showed

Table 4. Temperature rise after antimicrobial photodynamic therapy (a-PDT)

	BL (30 J/cm^2)				
RB final concentration (μg/mL)	0	0.16	1.6	16	160
Temperature rise (ºC)	3.8 ± 0.1	4.0 ± 0.1	4.3 ± 0.2	4.6 ± 0.2	7.9 ± 0.4
		*			
			**		
				***	

Results are given as mean ± standard deviation.
BL, blue light-emitting diode; RB, rose bengal.
Statistically significant differences among experimental groups ($n = 3$) are shown as *($p < 0.05$), **($p < 0.001$) and ***($p < 0.0001$).

that only RB enhances the a-PDT effect. Thus, the a-PDT effect is influenced by the photosensitizing ability, which, in turn, is dependent on the chemical structure of the dye agent and not on the complete matching wavelength of dye and light. In the present study, an a-PDT effect was obtained when BL and RB were combined; however, for BL, a more suitable dye with an absorption wavelength that matches well with the emission wavelength of BL might enhance the efficiency of the photochemical reaction.

The mechanism of killing of a-PDT is hypothesized to be as follows. Upon light irradiation, the dye is excited, leading to the production of cytotoxic reactive oxygen species and resulting in cell death (17). Among all the reactive oxygen species, singlet oxygen is highly cytotoxic in PDT (37,38), indicating that the greater the amount of singlet oxygen produced, the more effectively undesired cells, such as cancer cells and bacteria, are killed. However, quite recently, Ishiyama *et al*. (36) and Kato *et al*. (35) demonstrated that the ability of the dye to produce reactive oxygen species, such as singlet oxygen, is not the only factor contributing to the a-PDT effect but that the penetration efficiency of dye into the bacterial cells is also a factor. In their studies on singlet oxygen production among the red dyes, PB produced the highest amount and RB produced the lowest amount. However, RB was incorporated into the bacterial cells more efficiently compared with the other two photosensitizers, which probably led to the highest bactericidal activity observed for RB (35,36).

In the present experimental condition, the a-PDT effect increased in a dye concentration-dependent manner up to 16 μg/mL; however, the a-PDT effect decreased at the highest concentration of dye (160 μg/mL). This reverse phenomenon could be explained as follows: the 160 μg/mL concentration was so dark that the power of BL would have been reduced by being absorbed during light penetration into the red-colored solution, resulting in a decrease in the photochemical reaction.

Heat generation after light irradiation is one of the main drawbacks of light therapy (11). In the present study, in the BL-only group, there was a temperature elevation of 3.8–8.7°C at 30–90 J/cm^2. When using 30 J/cm^2 BL (30 s) in combination with RB up to a concentration of 16 μg/mL, the elevation was within 4.5°C. It is speculated that this minimal temperature elevation for a short time-period would not have any negative effect on the periodontium. In particular, if applied *in vivo*, the degree of temperature elevation would be less compared with that of the present experiment, which was performed at room temperature. Although there was a temperature elevation of 7.8°C when using 160 μg/mL of RB, this concentration is not useful because the antimicrobial effect was lower compared with the a-PDT using 16 μg/mL of RB. This result also suggests that temperature elevation is not directly associated with the death of bacteria.

RB is currently used in the medical field. It has been employed as a diagnostic tool for dry eye (39) and has been under investigation in clinical trials as a candidate for cancer therapy (40,41). Its role in photoactivated tissue bonding, a novel tissue-sealing technology that immediately cross-links proteins between tissues planes, thereby sealing on a molecular scale, has also been studied (42). In Japan, RB is approved as a food additive for red coloration. Regarding the concern of the negative effect of BL on the periodontium, a previous *in-vivo* study showed no tissue alteration following BL irradiation at 144 J/cm^2 (20 min) on rat palatal mucosa. Interestingly, tissue healing induced by LED biostimulation has also been demonstrated (43). However, further detailed studies are necessary to clarify the toxicity of BL or RB, alone, and in combination, on the periodontium in the present experimental condition before clinical application. In addition, in this study all experiments were conducted using one laboratory strain of *P. gingivalis* (ATCC 33277). The experiments should be conducted in clinical isolates and in other bacteria to determine the effects of BL and a-PDT using red dye in the real clinical situation.

Basically, the light and the dye for a-PDT should ideally not exert any toxic effect; however, if both light and dye independently possess some antimicrobial effect, a-PDT could be more effective and advantageous for disinfection in the complex environment of highly advanced deep periodontal pockets with bacterial invasion into the soft tissues. Both BL and RB have shown some antimicrobial effects on *P. gingivalis* ATCC 33277 in our study. Therefore, this novel a-PDT system can be considered as a viable alternative tool for pocket disinfection in the treatment of periodontal and peri-implant diseases. Also, this a-PDT system could effectively complement mechanical plaque control if a toothbrush is combined with BL after applying a red-colored mouth rinse.

Conclusion

Within the limits of this study, BL has an antimicrobial/growth-inhibiting effect on *P. gingivalis*, and a-PDT, using the combination of BL and RB, shows promise as a new treatment modality for bacterial elimination in periodontal therapy.

Acknowledgements

We would like to express our special thanks to Dental Techica, Osaka, Japan and CMS Dental, Copenhagen, Denmark for providing technical support for this study. This study was supported in part by a grant from the Global Center of Excellence Program for International Research Center for Molecular Science in Tooth and Bone Diseases at Tokyo Medical and Dental University from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Scientific Research (C) (22592308) to A. A. and a Grant-in-Aid for Young Scientists (B) (24792321) to Y. T. from the Japan Society for Promotion of Science. The findings of this study were presented at the 53rd Autumn Meeting of the Japanese Society of Periodontology (JSP) in September 2010 at Takamatsu, Kagawa, Japan; the 54th Autumn Meeting of the JSP in September 2011 at Shimonoseki, Yamaguchi, Japan; and the 97th Annual Meeting of American Academy of Periodontology in November 2011 at Miami, Florida, USA.

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ORIGINAL ARTICLES

Effect of antimicrobial photodynamic therapy using rose bengal and blue light-emitting diode on *Porphyromonas gingivalis in vitro*: Influence of oxygen during treatment

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Aims: A combination of rose bengal (RB) and blue LED (BL) has emerged as a new technical modality for antimicrobial photodynamic therapy (a-PDT). The purpose of this study was to clarify the influence of oxygen on the antimicrobial effect of RB + BL treatment on *Porphyromonas gingivalis in vitro*.
Materials and Methods: *P. gingivalis* cells were treated with RB, BL (450–470 nm; 1 W/cm^2, 5 s), or RB + BL under anaerobic/aerobic conditions. Cells were incubated anaerobically, and the cell density ($OD_{600\ nm}$) was measured after 6–48 h. Additionally, cells were cultured anaerobically on blood agar plates for 9 days, and the resulting colonies were observed. Bacterial growth within 1 h of aerobic RB + BL treatment was examined, and RNA degradation due to anaerobic/aerobic RB + BL treatment was measured after 3 h of culture.
Results: Under anaerobic conditions, RB + BL significantly suppressed bacterial growth after 18 h; however, the growth after 48 h and the number of colonies after 9 days were similar to those of the untreated control. RNA degradation in the anaerobic-treatment group was not significantly different from that in the control. Under aerobic conditions, RB + BL immediately affected bacterial growth and completely inhibited growth for up to 48 h. Few colonies were detected even after 9 days of culture, and RNA was completely degraded.
Conclusions: Unlike the bacteriostatic effect of anaerobic treatment, aerobic RB + BL treatment may have a bactericidal action via a-PDT effect, resulting in the destruction of RNA and bacterial cells within a short period.

Keywords: photodynamic therapy · LED · rose bengal · periodontal disease · *Porphyromonas gingivalis*

Introduction

Periodontal diseases arise from inflammatory conditions around the tooth supporting periodontal tissues. Inflammation primarily occurs as a response to chronic infections against periodontopathic bacteria. The management of periodontal diseases is based on the removal of these bacteria, which is mainly achieved by mechanical removal using hand instruments, together with chemical substances. However, it is difficult to completely remove pathogenic agents with these conventional methods. In addition, chemotherapy using antibiotics occasionally causes side effects, including the development of drug-resistant bacteria [1]. Therefore, novel noninvasive/nonsurgical methods

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Received date: August 23rd, 2016
Accepted date: October 20th, 2016

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need to be developed to address these issues in the aging population.

Conventionally, light energy, including lasers, has been applied as a treatment strategy to destroy infectious pathogenic microorganisms [2], and antimicrobial photodynamic therapy (a-PDT) based on a photochemical reaction was introduced in dental treatments [3, 4]. a-PDT is a non-invasive treatment procedure that uses low-level light energy to activate a photosensitizing agent; generally, the combination of a red-colored light and a blue dye has been applied in the field of periodontal therapy [3]. On the other hand, considering the wavelength of light, blue light itself exhibits an antimicrobial effect, which may be advantageous in a-PDT [5-7].

Recently, the combination of a red dye agent and blue light emerged as a new technical modality for a-PDT [8, 9]. In a previous study, we demonstrated that a-PDT using a red dye, rose bengal (RB), and blue LED (BL) strongly inhibited colony formation of the representative periodontopathic bacterium, *Porphyromonas gingivalis*, *in vitro*, and that a-PDT using RB and BL shows promise as a new technical modality for bacterial elimination in periodontal therapy [10]. a-PDT has three components: visible harmless light, a nontoxic photosensitizer, and oxygen [11, 12]; further, it is well known that a-PDT does not induce resistance in microorganisms [3]. Photosensitizing agents activated by light in the presence of oxygen generate singlet oxygen and/or superoxide anion, which are fatal for bacteria [13], and thus, the necessity and importance of oxygen during a-PDT is generally accepted [3, 4, 11, 12]. However, the influence of oxygen during a-PDT procedure, in particular, on the antibacterial effect of RB + BL treatment has not been clarified precisely, and the individual effect of each component (RB and BL) remains unclear, especially in the case of anaerobic bacteria such as *P. gingivalis*.

Therefore, in the present study, we treated *P. gingivalis* suspension with RB, BL, or RB + BL in the presence/absence of oxygen to precisely clarify the influence of oxygen on the outcome of a-PDT.

Materials and Methods

Bacteria and culture conditions

P. gingivalis ATCC 33277^T was grown in tripticase soy broth supplemented with phytone peptone (5 mg/mL), yeast extract (5 mg/mL), NaCl (5 mg/mL), cysteine (0.1%), hemin (5 mg/mL), and menadione (1 μg/mL) at 37 °C in an anaerobic chamber (Forma Scientific 1024, Ontario, Canada) containing 80% N_2, 10% H_2, and 10% CO_2. The chamber was impenetrable to light. Before initiating bacterial growth, fresh growth medium and saline were incubated anaerobically for 3 days to remove any residual oxygen.

Preparation of the photosensitizer

RB (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), often used as a dental plaque tester in the clinical setting, was employed as a photosensitizer. RB solution was freshly prepared in deaerated sterile physiological saline before use. First, the effect of RB concentration on *P. gingivalis* growth was investigated. *P. gingivalis* was grown to the mid-log phase. The bacterial suspension (216 μL) and RB (24 μL) at 0.01%, 0.005%, or 0.001% (wt/wt) were added into the wells of a 96-well titer plate (total volume: 240 μL/well; final RB concentration: 0.001%, 0.0005%, or 0.0001% [wt/wt]). Bacterial suspension mixed with saline (24 μL) was used as a control. After treatment, all the suspensions were incubated at 37 °C in an anaerobic chamber, and the cell density ($OD_{600\ nm}$) was measured every 1 h for up to 12 h.

Light apparatus

A high-power blue-colored LED (BL; a device modified from Flashmax 2^{TM}, CMS Dental, Denmark; wavelength 425–500 nm, power density 4 W/cm^2) was used as the non-laser light source in this study. This light source is also used for composite resin polymerization in dental caries treatment. The apparatuses were fixed onto a stand within an anaerobic chamber, so that the light-emitting end (6.8 mm in diameter) of the LED apparatus was positioned to match the top entrance of a well (6.8 mm in diameter) in a 96-well titer plate during irradiation.

BL irradiation was performed with the light-emitting end in direct contact with the entrance of the well. The distance between the top surface of the bacterial suspension and the light-emitting end was 4.5 mm, and the depth of the mixed bacterial suspension was 7.5 mm. The actual energy level on the surface of the mixed solution was approximately 480 mW (power meter: NOVA II, Optical Metrology Ltd., Jerusalem, Israel; detection head: PD 300, 3 W), and the power density was calculated to be approximately 1.5 W/cm^2.

LED irradiation

P. gingivalis was grown to the late-log phase. The bacterial suspension ($OD_{600\ nm}$ = 0.1, 216 μL) and 0.001% RB (24 μL) or saline (24 μL) were added in the wells of a 96-well titer plate (total volume: 240 μL/well; final RB concentration: 0.0001% [wt/wt]) and irradiated with BL

for 5 s or left as is (non-irradiated) under aerobic/anaerobic conditions. The treated samples were incubated at 37 °C for 6, 12, 18, 36, and 48 h in an anaerobic chamber that was impenetrable to light, and the cell density ($OD_{600\ nm}$) was measured.

In addition, after 18 h, each bacterial suspension was diluted 1,000 times, and 100 µL of each diluted suspension was inoculated on tripticase soy agar plates (supplemented with 50 mL/L horse blood, 5 mg/L hemin, and 50 µg/L vitamin K1) and anaerobically incubated at 37 °C for 9 days. Furthermore, in order to examine the effect of aerobic RB + BL treatment on bacterial growth over a short period, a high concentration of the bacterial suspension ($OD_{600\ nm}$ = 0.8) was used at the start of the experiment, and the cell density ($OD_{600\ nm}$) was measured every 10 min for up to 60 min after treatment.

Preparation of total RNA

Stabilization of bacterial RNA, bacterial destruction, and RNA purification were carried out as described previously [7]. After treatment with BL + RB under anaerobic/aerobic conditions, *P. gingivalis* was incubated for 3 h anaerobically. The cell suspensions were mixed with RNAprotect™ Bacteria Reagent (Qiagen, Tokyo, Japan) to stabilize the bacterial RNAs. Total RNAs were prepared using TRIzol™ reagent (Invitrogen, Carlsbad, CA) and the PureLink™ RNA Micro Kit (Invitrogen) according to the manufacturer's instructions. Bacterial disruption was performed using a FastPrep FP120 Instrument (Qbiogene, Carlsbad, CA). The RNA quality was examined, and the RNA integrity number (RIN: 1–10 values) was tested for RNA degradation by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with an RNA 6000 Nano LabChip Kit (Agilent).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). One-way analysis of variance followed with the Tukey-Kramar HSD procedure as a post-hoc test was applied for comparing cell densities of test and control groups. In addition, unpaired *t*-test with Bonferroni correction was performed to determine whether the cell densities in the anaerobic and aerobic conditions differed. Values of $p < 0.05$ were considered significant.

Results

Effect of RB on bacterial growth

The OD of *P. gingivalis* suspension containing 0.01% RB solution (final concentration: 0.001%) reduced gradually after treatment, suggesting the occurrence of growth inhibition, and the increase of OD was significantly suppressed upon the addition of 0.005% RB (final concentration: 0.0005%), compared with the control. The OD of the suspension containing 0.001% RB (final concentration: 0.0001%) was not significantly different from that of the control, although a very slight effect on growth was observed compared with the control **(Fig. 1)**.

Effect of a-PDT on bacterial growth

Although the OD of *P. gingivalis* suspension treated anaerobically with 0.001% RB + BL was elevated for up to 18 h post-treatment, it was still clearly suppressed in comparison with the non-treated control, while the OD of the group treated aerobically with RB + BL remained low and unchanged during the observation period, indicating that bacterial growth was completely

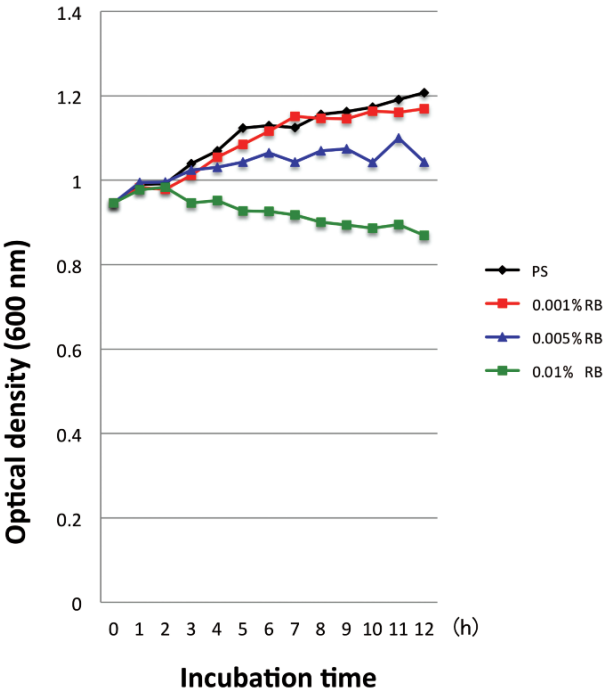

Fig. 1: The effect of rose bengal (RB) addition on bacterial growth.
P. gingivalis was grown to the mid-log phase. The bacterial suspension (216 µL) and 0.001, 0.005, or 0.01 % (w/w) RB (24 µL) were added into the wells of a 96-well titer plate (total volume, 240 µL/well), and the plates were incubated in an anaerobic chamber. The cell density ($OD_{600\ nm}$) was measured every 1 h for up to 12 h. PS, physiologic saline.

inhibited **(Fig. 2)**. Eighteen-hours after treatment, the ODs of the groups treated with either RB or BL alone were lower than that of the control group. Furthermore, significantly lower ODs were observed in the group treated with RB + BL under anaerobic/aerobic conditions ($p < 0.0001$), and the decreasing trend was more noticeable in the groups treated under aerobic conditions than in groups treated under anaerobic conditions ($p < 0.001$; **Fig. 3**).

Next, we investigated whether the growth inhibition effect of these treatments was bacteriostatic or bactericidal in action. To clarify this aspect, culture of *P. gingivalis* suspension was continued until 48 h after treatment. In addition, the treated bacterial suspension

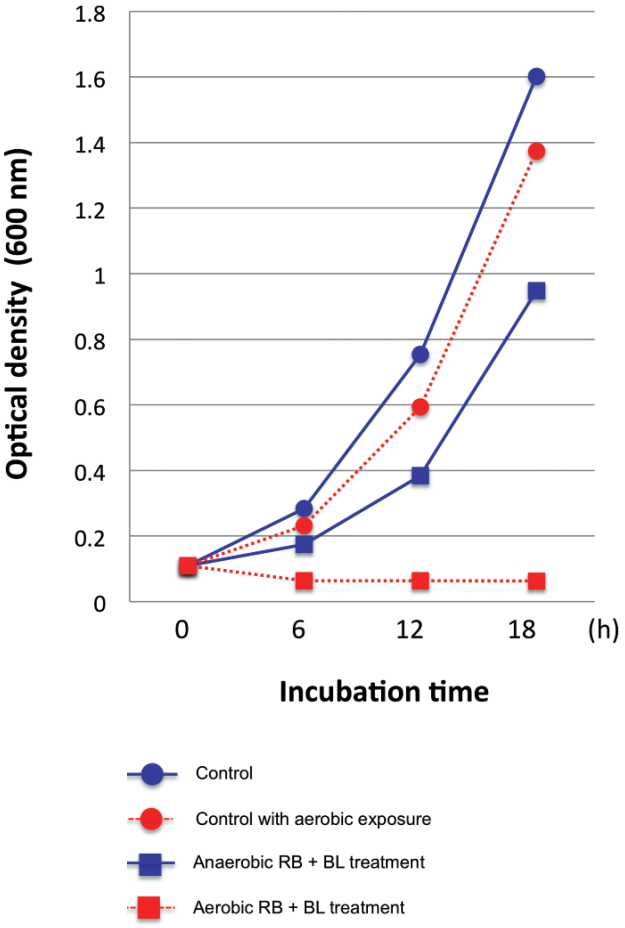

Fig. 2: *P. gingivalis* growth curves after RB + blue LED (BL) treatment under anaerobic/aerobic conditions. *P. gingivalis* suspension ($OD_{600\ nm}$ = 0.1) was treated with RB + BL anaerobically (square, solid line) or aerobically (square, dashed line) and then incubated in an anaerobic chamber. Non-treated control (circle, solid line) and control with aerobic exposure for the same period as that of the RB + BL treatment group (circle, dashed line) were also incubated anaerobically. The cell density ($OD_{600\ nm}$) was measured as an indication of the bacterial growth at 6, 12, and 18 h after treatment.

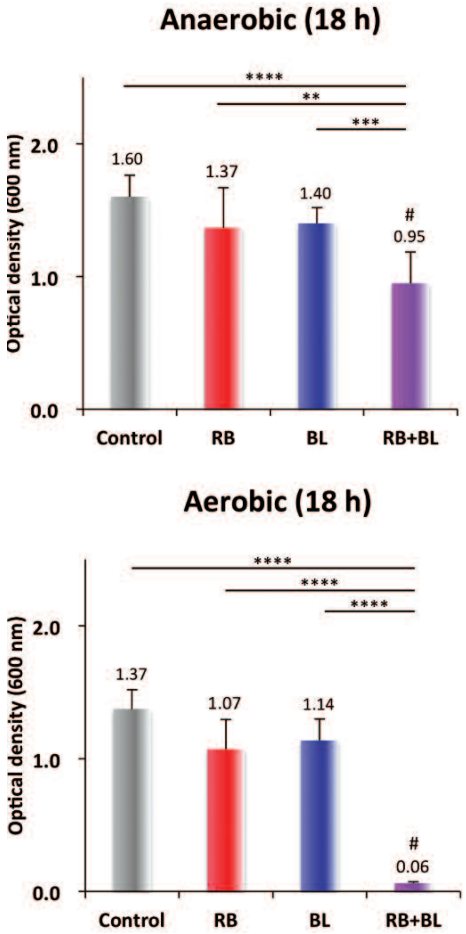

Fig. 3: *P. gingivalis* growth at 18 h after various treatments. *P. gingivalis* was treated with RB, BL, or RB + BL anaerobically or aerobically and then incubated in an anaerobic chamber. Control with aerobic exposure for the same period as that of the RB + BL treatment group was also incubated anaerobically. The cell density ($OD_{600\ nm}$) was measured at 18 h after treatment. Data are presented as the mean ± standard deviation (SD; n = 9). **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ (one-way analysis of variance [ANOVA] followed by Tukey-Kramar HSD procedure as post-hoc). #: $p < 0.001$ (between the aerobically and anaerobically treated RB + BL groups, unpaired t-test with Bonferroni correction).

was inoculated on a blood agar plate and cultured for 9 days. All groups, except for the aerobically treated RB + BL group reached the stationary phase at 36 h post-treatment. The lowest OD value was observed in the group treated aerobically with RB + BL (data not shown), and therefore, it was monitored until 48 h after treatment, and the same results, with no change in the aerobic RB + BL treatment, were confirmed at 48 h post-treatment **(Fig. 4)**. On the blood agar plates, after 9 days, few colonies were observed in the aerobic RB + BL treatment group, compared with the non-treated control and the anaerobic RB + BL treatment group, both of which showed numerous colonies **(Fig. 5)**.

To further verify the bactericidal effect of a-PDT,

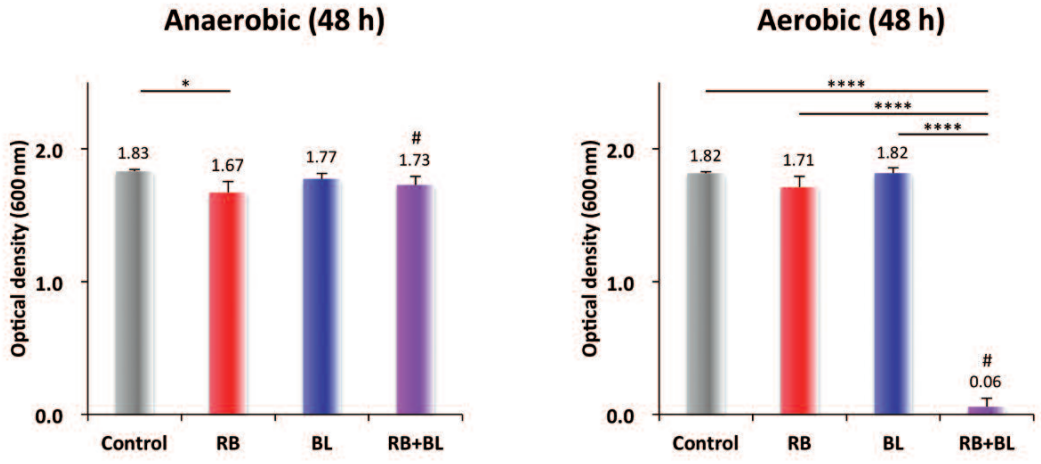

Fig. 4: *P. gingivalis* growth at 48 h after various treatments.
P. gingivalis was treated with RB, BL, or RB + BL anaerobically or aerobically and then incubated in an anaerobic chamber. The cell density ($OD_{600\ nm}$) was measured at 48 h after treatment. Data are presented as the mean ± SD (n = 3). *: $p < 0.05$, ****: $p < 0.0001$ (one-way analysis of variance [ANOVA] followed by Tukey-Kramar HSD procedure as post-hoc). #: $p < 0.001$ (between the aerobically and anaerobically treated RB + BL groups, unpaired t-test with Bonferroni correction).

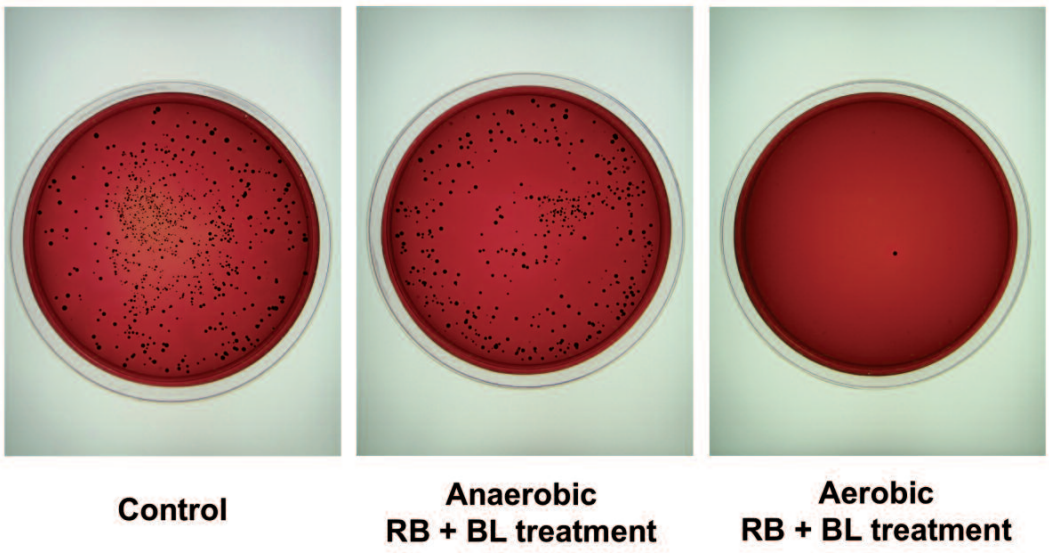

Fig. 5: Colony formation on a blood agar plate after 9 days.
P. gingivalis was treated with RB + BL anaerobically or aerobically and then incubated for 9 days in an anaerobic chamber. Non-treated control was also incubated anaerobically.

we prepared a high-density *P. gingivalis* suspension ($OD_{600\ nm}$ = 0.8) and measured the short-term change of OD values after aerobic RB + BL treatment **(Fig. 6)**. The OD reduced immediately after treatment and reached a plateau with a value of under 0.2 after 40 min, suggesting destruction of cells, in contrast to the increasing tendency of the OD in the non-treated control (where the OD finally reached 1.0).

Investigation of bacterial viability

Degradation of total RNAs was examined by determining the RIN using samples prepared after 3 h of incubation post treatment with RB + BL. The RNA fragments were analyzed by microcapillary electrophoresis, and typical peaks for 23S, 16S, and 5S rRNA were confirmed in the control (A) and anaerobic RB + BL treatment (B) groups; however, no peaks of rRNAs were detected in the aerobic RB + BL treatment group (C; **Fig. 7**).

The RINs are valued from 1 to 10, with a value of 10 indicating a perfect RNA sample without any degradation products, whereas a value of 1 indicates a completely degraded sample [14]. In this study, the RIN values of the anaerobic non-treated control (A) and the anaerobic RB + BL treatment group (B) were 9.3 and 8.1, respectively. The RIN value of the aerobic RB + BL treatment group (C) could not be measured because of excessive degradation.

Discussion

P. gingivalis is a black-pigmented, Gram-negative anaerobic bacterium believed to be an important etiological agent in adult periodontal disease [15, 16]. Many virulence factors, such as fimbriae, protease, hemagglutinins and capsular polysaccharide, allow this organism to cause disease [15]. This bacterium is known to mediate the coaggregation of various oral Gram-positive and Gram-negative bacteria. In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilms containing multiple species, which colonize specific surfaces and result in interactive nutritional chains. The regulation of this bacterium may thus lead to biofilm regulation, and may thus serve as a strategy for improving or maintaining gingival health.

Lately, a-PDT has been applied in bacterial elimination for the treatment of periodontal pockets in periodontal therapy [3, 4]. Currently, a-PDT in combination with blue dye agents, such as methylene blue and toluidine blue, and red diode laser or LED, is employed [3].

Recently, we focused on the antimicrobial effect of blue lights [5, 6], and analyzed the suitability of RB as a photosensitizer [17, 18] and their combined effects on a-PDT [8]. RB has been approved as a food additive for red coloration in Japan and is one of the dyes used for dental plaque disclosing. Ishiyama et al. [17] and Kato et al. [18] showed that, among the three red dyes

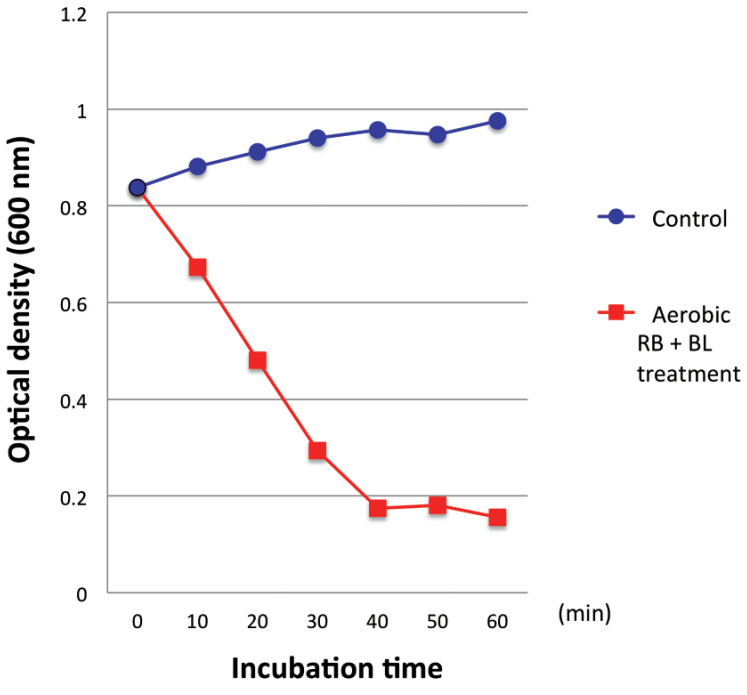

Fig. 6: *P. gingivalis* growth curves after aerobic RB + BL treatment within 1 h. The bacterial cell density ($OD_{600\ nm}$) at experiment initiation was adjusted to 0.8, and the growth change within 1 h after aerobic RB + BL treatment was investigated in comparison with the non-treated control.

employed as plaque disclosing agents (erythrosine, phloxine, and RB), RB exhibited the highest a-PDT activity. BL is usually employed in daily dental practice as a photopolymerization device of resin materials used for dental caries treatment. Our group had previously investigated the effect of BL on *P. gingivalis* in anaerobic condition and demonstrated that BL has a growth-inhibiting effect on *P. gingivalis* owing to a bacteriostatic action mediated by the suppression of genes associated with chromosomal DNA replication and cell division at the transcriptional level [7]. Additionally, we reported the excellent antimicrobial

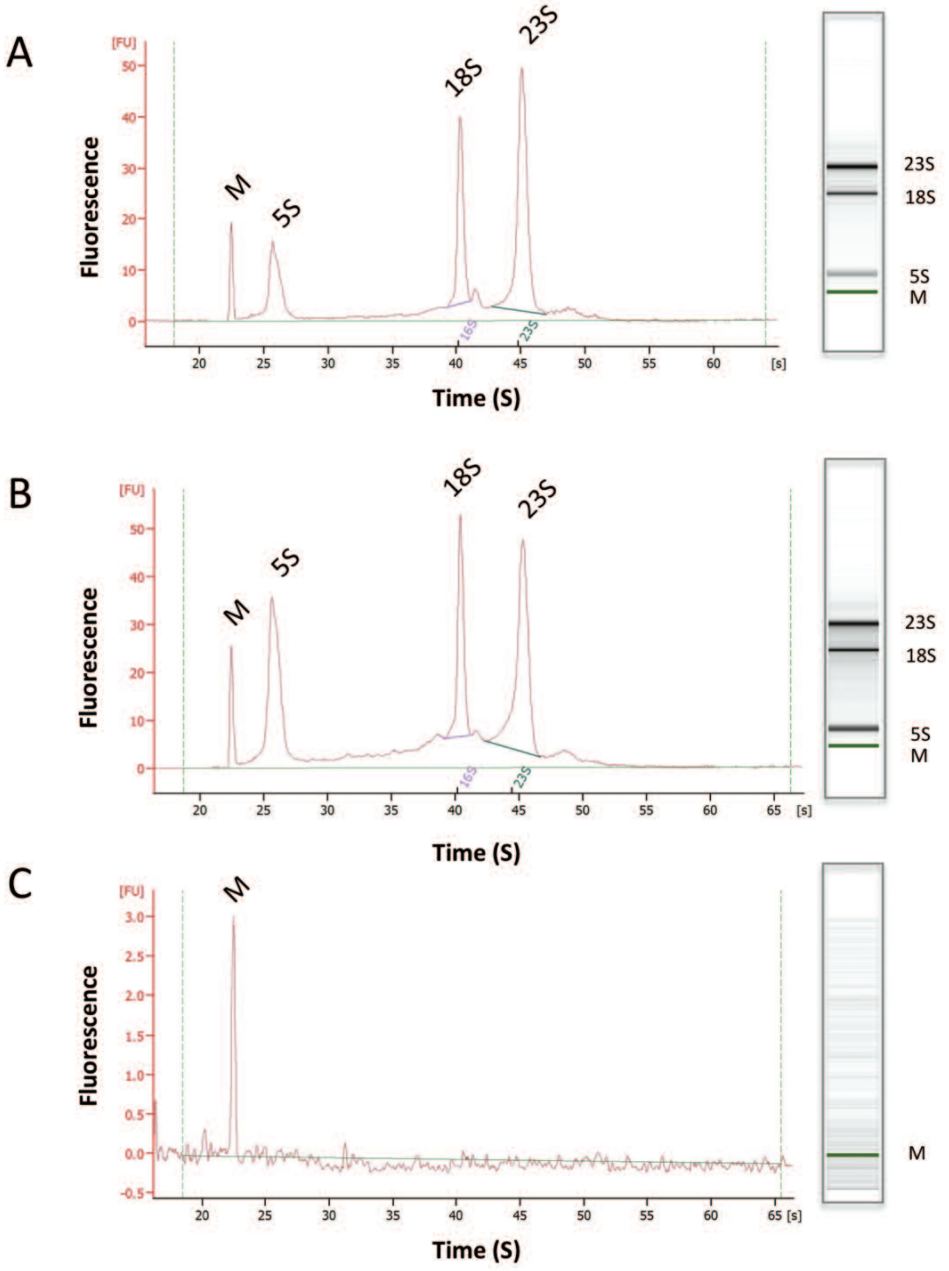

Fig. 7: RNA fragment patterns obtained by microcapillary electrophoresis and their predicted electropherograms on an agarose gel.
After purification of *P. gingivalis* total RNA from the control (anaerobic, non-treated; **A**), anaerobic RB + BL treatment (**B**) and aerobic RB + BL treatment (**C**) groups, the RNA quality was examined by using an Agilent 2100 bioanalyzer. M, maker; 5S, 5S rRNA; 16S, 16S rRNA; 23S, 23S rRNA.

effect of a-PDT using a combination of RB and BL against *P. gingivalis* under aerobic conditions [10]. However, the precise effects of each component, as well as the combined effect under the presence and absence of oxygen, remained unclarified.

Therefore, in the present study, the antimicrobial effects of RB, BL, or RB + BL on *P. gingivalis* under anaerobic and aerobic conditions were investigated, and the influence of oxygen during the a-PDT procedure with a combination of BL and RB was clarified. RB or BL alone inhibited bacterial growth to similar extents, and the growth inhibition was more significant upon combined treatment with RB + BL than with RB or BL individually. Moreover, the effect was more significant under aerobic conditions than under anaerobic conditions at 18 h post-treatment. In the anaerobic condition, although RB + BL application significantly retarded *P. gingivalis* growth for up to 18 h after treatment, the bacterial growth continued and recovered to the same level as that of the non-treated control after 48 h, suggesting that the effect may be temporal bacteriostatic action. On the other hand, under aerobic conditions, RB + BL application did not increase the cell density for up to 48 h, and very few bacterial colonies were formed even after 9 days. Thus, aerobic RB + BL treatment almost completely inhibited bacterial growth, suggesting bactericidal action.

In order to confirm this finding, we performed the following two experiments. First, we investigated the change in bacterial growth after irradiation under aerobic conditions by using a high-density bacterial suspension. Secondly, we extracted RNA from *P. gingivalis* after RB + BL treatments and examined the quality of RNA by determining the RIN values [14]. Immediately after RB + BL application under aerobic conditions, the cell density decreased rapidly and reached a plateau (<2.0) at 40 min post-treatment (the value of 2.0 may be attributable to the bacterial debris remaining after bacterial destruction). This suggested that the destruction of bacterial cells was completed within 40 min after treatment. Furthermore, after aerobic RB + BL application, the primary peaks of rRNA were completely diminished, suggesting that the bacterial cells may have lysed completely. On the other hand, the quality of RNA following anaerobic RB + BL application showed no remarkable change in comparison with the non-treated control. Thus, it may be concluded that the effect of RB + BL treatment under anaerobic conditions was bacteriostatic, similar to the effect of RB or BL treatment individually, and that the effect of the aerobic treatment was bactericidal, probably because the a-PDT effect occurred in the presence of oxygen.

Regarding the current clinical applications of a-PDT in periodontal pocket therapy, Sgolastra et al. [19] reported that a-PDT used adjunctive to conventional scaling and root planning (SRP) provided short-term benefits in term of probing depth reduction and attachment level. In addition, Sculean et al. [20] reported that, in patients with chronic periodontitis, the combination of SRP and a-PDT may result in substantial short-term clinical improvements, as evidenced by probing depth and bleeding on probing reductions compared with SRP alone. Therefore, the positive effect of a-PDT is still limited to a short term, suggesting that the antimicrobial effect may be clinically weak.

Various reasons may account for the minor clinical effects of a-PDT, such as the power and irradiation time of lights, concentration of dyes, and combination of dyes and lights, etc. Periodontal pockets become more anaerobic with the deepening of the pockets [21], and thus, the oxygen-deficient conditions could potentially be one of the causes for the lowered effectiveness of a-PDT in the pockets. The original amount of oxygen contained in the dye solution might also be insufficient for effective a-PDT. Therefore, considering the results of the present study, intentionally supplying oxygen into the periodontal pockets by modifying the dye solution during the a-PDT procedure might be an effective strategy for enhancing the antimicrobial effects of PDT in periodontal pockets. On the other hand, in the case of preventive application of a-PDT, such as in dental plaque control [22], there is no concern about the presence of oxygen, since a-PDT is performed in the oral cavity under aerobic conditions. Thus, further analysis is required to determine the optimal oxygen concentration in the clinical application of a-PDT in the periodontal pockets.

In conclusion, the effect of RB + BL treatment under anaerobic conditions was bacteriostatic while that under aerobic condition became bactericidal by exerting the a-PDT effect. Furthermore, after RB + BL application under aerobic conditions, destruction of RNA as well as bacterial cells occurred within a short period.

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[Acknowledgements]
We are grateful to Dr. Sayaka Katagiri, Department of Periodontology, TMDU for statistical analysis and Ms. Hatsumi Furutani, Dental Technica Inc., Osaka, Japan for her technical advice on LED application. This research was supported by JSPS KAKENHI (grant numbers 25463212 and 16K11825 to A.A., and 25293424 to Y.I.).

Photobiomodulation, Photomedicine, and Laser Surgery
Volume XX, Number XX, 2019
© Mary Ann Liebert, Inc.
Pp. 1–10
DOI: 10.1089/photob.2018.4514

Original Research

The Effects of Ultraviolet Light-Emitting Diodes with Different Wavelengths on Periodontopathic Bacteria *In Vitro*

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Abstract

Objective: The aim of this study was to examine effects of recently developed ultraviolet light-emitting diodes (UV LEDs) wavelengths on *in vitro* growth and gene expression of cultural periodontopathic bacteria, and on viability of experimental gingival fibroblasts.
Materials and methods: *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, and *Streptococcus oralis* were irradiated by UV LEDs (265, 285, 310, 365, and 448 nm) at 600 mJ/cm^2 and grown anaerobically *in vitro*. The colony forming units were counted after 1 week. Cell morphology was observed using a scanning electron microscope (SEM). Quantitative real-time polymerase chain reaction was performed to investigate gene expression changes by 310 nm irradiation. Viability of the irradiated human gingival fibroblasts was evaluated using WST-8 assay.
Results: Both 265 and 285 nm resulted in the complete death of bacteria and fibroblasts, whereas 310 nm caused partial killing and suppression of bacterial growth and much less damage to the fibroblasts *in vitro*. Both 365 and 448 nm resulted in no significant change. SEM showed that *P. gingivalis* cells gradually degraded from day 2 or 3 and were severely destructed on day 5 for 265, 285, and 310 nm. The 310 nm irradiation transiently suppressed the transcripts of SOS response- and cell division-relative genes.
Conclusions: Both 265 and 285 nm may induce powerful bactericidal effects and severe fibroblast phototoxicity, and 310 nm may induce partial killing or growth suppression of bacterial cells with much less fibroblast phototoxicity. UV lights may have potential for bacterial suppression, with situations dependent on wavelength, in periodontal and peri-implant therapy.

Keywords: UV LED, periodontopathic bacteria, human gingival fibroblasts

Introduction

Periodontal diseases represent inflammation around the tooth-supporting periodontal tissues that is mainly initiated by chronic infections of periodontopathic bacteria.[1–3] The purpose of periodontal therapy is to arrest infection and inflammation of periodontal tissues.[3,4] However, conventional periodontal therapies, such as mechanical therapy and chemotherapy, do not completely eradicate bacteria.[5,6] Antibiotic therapy also has various disadvantages such as allergy, difficulty in maintaining therapeutic concentrations in periodontal pockets, and occurrence of bacterial resistance.[7–9]

Lasers and photodynamic therapy are beneficial in bacterial elimination, without some of the above limitations.[10,11] Recent studies have suggested the clinical efficacy of laser therapy as an adjunct to scaling and root planing, and it has been reported to produce improved clinical outcomes in the treatment of periodontitis.[12–15] However, laser devices are costly, and cause thermal denaturation and carbonization of soft and hard tissues.[16] Antimicrobial photodynamic therapy (a-PDT) using a combination of light and photosensitizer has also been employed for the treatment of infectious diseases including periodontitis.[17,18] On the other hand, ultraviolet light (UV) irradiation alone has been demonstrated to possess

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bactericidal effects and has been employed for sterilization in medicine.[19,20]

UV irradiation (wavelength: 100–400 nm), an electromagnetic irradiation, can be subdivided into UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm).[21] Recently, UV irradiation has been used for treating skin diseases such as psoriasis and atopic dermatitis, especially UVB around 310 nm, which has a more beneficial therapeutic effect and less side effects for human cells compared with other UV wavelengths.[22–24] The therapeutic effects of UV wavelengths may also be applicable in the treatment of periodontitis and peri-implantitis. However, little is known about the effects of UV wavelengths on periodontopathic bacteria and periodontal tissues[25,26] and only one previous *in vitro* study described the effects of UV light-emitting diodes (LEDs) on oral bacteria.[26] Additionally, compact, portable, easy-to-use UV LED devices are being developed as convenient, cost-effective, light delivery methods for clinical use.[26] Therefore, UV LEDs may be considered for clinical application in periodontal and peri-implant treatment.

We hypothesized that antibacterial effects of UV LEDs would vary greatly depending on the wavelengths. Also, we wanted to evaluate the influence of UV LEDs on periodontal tissues in view of their possible application in periodontal therapy. Thus, the aim of this study was to examine effects of different UV LED wavelengths on cultured periodontopathic bacteria in terms of *in vitro* bacterial growth, cell morphology, gene expressions, and on the viability of experimentally prepared human gingival fibroblasts (HGF-1).

Materials and Methods

LED apparatus

An LED apparatus with UV light 265, 285, 310, 365, and visible blue light 448 nm (Nikkiso Giken Co., Ltd., Ishikawa, Japan) was employed. The apparatus was fixed onto a stand so that the light-emitting end was positioned to match the top entrance of a well (6.8 mm in diameter, 0.32 cm^2) in a 96-well flat-bottom titer plate (Falcon®; Corning Inc., Corning, NY) during irradiation (Fig. 1). Irradiation was performed for ~1 min at a total energy density (ED) of ~600 mJ/cm^2. ED was adjusted by selecting a specific power density, and suitable irradiation time, for each wavelength [598.5 mJ/cm^2 (9.50 mW/cm^2 and 63 s) for 265 nm; 601.0 mJ/cm^2 (9.39 mW/cm^2 and 64 s) for 285 nm; 599.4 mJ/cm^2 (9.99 mW/cm^2 and 60 s) for 310 nm; 602.1 mJ/cm^2 (9.87 mW/cm^2 and 61 s) for 365 nm; and 602.3 mJ/cm^2 (9.56 mW/cm^2 and 63 s) for 448 nm], as shown in Table 1.

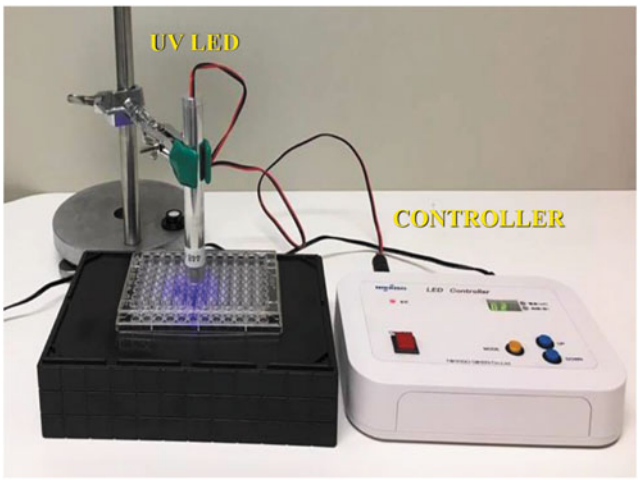

FIG. 1. A UV LED and a power controller. UV LED, ultraviolet light-emitting diode.

Bacteria and culture conditions

Porphyromonas gingivalis ATCC 33277 and *Prevotella intermedia* ATCC 25611 were grown in brain heart infusion (BHI; Difco Laboratories, Franklin Lakes, NJ) and on the agar plate supplemented with 5% (v/v) horse blood as previously described.[27] *Fusobacterium nucleatum* ATCC 25586, *Aggregatibacter actinomycetemcomitans* ATCC 43718, and *Streptococcus oralis* OMZ 607 were grown in BHI broth and on the agar plate supplemented with 5% or 10% (v/v) horse blood, respectively. All bacteria were incubated anaerobically at 37°C.

Preparation of bacterial suspension before LED irradiation

After washing with phosphate-buffered saline (PBS), bacterial cells were adjusted for optical density at 600 nm (OD_{600}) to 1.0 with PBS (~1×10^8 cells/mL) using an Eppendorf Biophotometer™ (Eppendorf AG, Hamburg, Germany). The bacterial cell suspension (100 μL) was added into a well of a 96-well microtiter plate and irradiated by UV LEDs aerobically and anaerobically. After irradiation, the cell suspension was transferred to a 1.5 mL tube, centrifuged, and the supernatant was removed. The resulting bacterial pellet was resuspended with new bacterial culture medium.

Colony forming units of various periodontopathic bacteria

P. gingivalis, *P. intermedia*, *F. nucleatum*, *A. actinomycetemcomitans*, or *S. oralis* suspension was irradiated by UV LEDs aerobically. Irradiated samples were spread on agar plates in triplicate, and incubated anaerobically 1 week at 37°C. Colony forming units (CFUs) were then calculated. The experiment was independently performed four times.

Before and immediately after UV irradiation, the *P. gingivalis* suspension temperature was measured at the center of the suspension at room temperature with a thermocouple device (Digital Thermometer IT-2000; Iuchi, Seieido, Osaka, Japan; $n = 10$).

In vitro P. gingivalis *growth*

P. gingivalis cells were irradiated with UV LEDs anaerobically or aerobically and the OD_{600} was measured at different time intervals up to 24 h ($n = 3$).

Scanning electron microscope analysis

Irradiated *P. gingivalis* was incubated anaerobically for 30 min, 1, 2, 3, and 5 days and fixed in 2.5% (v/v) glutaraldehyde for 2 h at 4°C. After washing three times with 0.1 M phosphate buffer (pH 7.2), they were postfixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol solutions for

Table 1. Ultraviolet Light Irradiation Parameters

Wavelength (nm)	*Power output (mW) 96 well area (0.32 cm²)*	*Irradiation time (seconds)*	*Total energy dose (~600 mJ/cm²)*	*Irradiation spot (mm)*	*Irradiation distance (mm)*
265	9.50 mW	63	598.5 mJ/cm²	6.8	7
285	9.39 mW	64	601.0 mJ/cm²	6.8	7
310	9.99 mW	60	599.4 mJ/cm²	6.8	7
365	9.87 mW	61	602.1 mJ/cm²	6.8	7
448	9.56 mW	63	602.3 mJ/cm²	6.8	7

10 min, dried at a critical point, and finally ion sputter coated. Morphological changes were observed with scanning electron microscope (SEM; S-4500; Hitachi, Tokyo, Japan). The experiment was repeated twice independently.

Quantitative real-time polymerase chain reaction

P. gingivalis was irradiated at 310 nm and incubated for either 5, 30, 60, 120, or 240 min, 1, 2, 3, or 5 days. RNA extraction and reverse transcription were performed as previously described.[27] RNA quality was determined by RNA integrity number (RIN: 1–10 values), a robust and reproducible RNA integrity calculation algorithm, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with an RNA 600 Pico Chips (Agilent Technologies).

Quantitative real-time polymerase chain reaction was performed as previously described,[28] for either 5, 30, 60, 120, or 240 min. Primers were designed from the NCBI database entry for *P. gingivalis* strain ATCC 33277 (NC 010729) and are shown in Table 2. All data were analyzed based on the comparative CT method. The resulting relative values represented the relative expression level of a given gene compared with the level in the nonirradiated control at a corresponding incubation time point ($n=4$).

Cytotoxicity on HGF-1

HGF-1 (ATCC CRL-2014, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), and 1% antibiotic-antimycotic mixture (Invitrogen) in a humidified atmosphere with 5% carbon dioxide (CO_2) at 37°C. The cells were seeded into 96-well plates (10,000 cells/well). At 48 h after seeding, medium was replaced with PBS, and HGF-1 cells were irradiated by UV LEDs. Treatments with chemical agents [0.025% benzalkonium chloride (BKC) and 3% hydrogen peroxide (H_2O_2)] for 20 s were used as positive control groups. The viability of irradiated HGF-1 was measured using WST-8 assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions at days 1 and 3 following irradiation. The ratio (%) of the cell viability of irradiated or treated HGF-1 relative to that of nonirradiated control was calculated ($n=3$).

Table 2. Polymerase Chain Reaction Primers

Gene ID	*Gene name*	*Direction*	*Primer sequence (5′-3′)*	*Size (bp)*	*Description*
PGN_0001	*dnaA*	Forward	TTTGGAGGGCAATTTCGTAG	124	Chromosome replication initiator
		Reverse	TGTCACCGTACCGGGATATT		
PGN_0041	*htpG*	Forward	AATGGAAAGACGGCAAGATG	91	Heat shock protein 90
		Reverse	TTGAGGTCAGCAGGCTTTTT		
PGN_0043	*ftsH*	Forward	GTAGGAGCCTCTCGTGTTCG	132	Cell division protein
		Reverse	CTCATCATTGCCGGAGAAA		
PGN_0631	*ftsZ*	Forward	TACCACCGATCCGGAGTTAG	111	Cell division protein
		Reverse	ACATTGCCAAGGTTGTCTCC		
PGN_1057	*recA*	Forward	GAATGGCCACGGAGAAGATA	137	DNA repair
		Reverse	GTAACCGCCTACACCGAGAG		
PGN_1126	*(UmuD)*	Forward	AGACAAGTCCCTTGAAGCGC	115	Error-prone repair: SOS-response transcriptional repressor UmuD homolog
		Reverse	GTTGGCCGGTTCGAGTAGAA		
PGN_1127	*(UmuC)*	Forward	TGGTTGTATCATCGCTCGCA	101	SOS mutagenesis and repair protein UmuC homolog
		Reverse	CCACATTGTGCCGACGAATC		
PGN_1208	*clpB*	Forward	AGAAACTGCCCCATGTATCG	129	ClpB protein
		Reverse	CTAGCACGATGTGCTCCAAA		
PGN_1451	*groES*	Forward	ATTCGGCCAAAGAGAAACCT	95	Chaperonin GroES
		Reverse	TACGGTGTCTCCTGCTTTGA		
PGN_1452	*groEL*	Forward	TAGAATTGGAGTGCCCGTTC	153	Chaperonin GroEL
		Reverse	CTCCTGCCGTAACGTTCTTC		
PGN_1786		Forward	GAGCCAAGCGCGTATCTATC	118	DNA polymerase III β chain
		Reverse	TCTTCAGCAGCCACAGAGAA		

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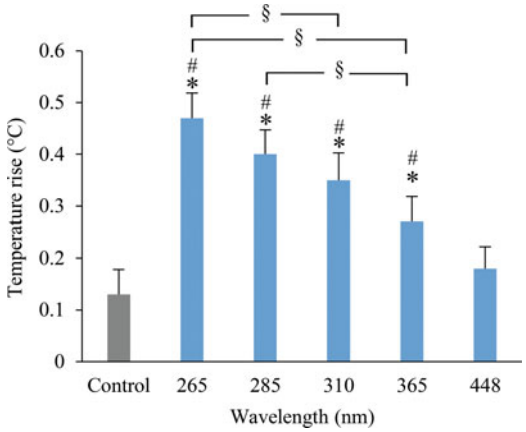

FIG. 2. Temperature change of *Porphyromonas gingivalis* suspension after UV irradiations. Data are presented as the mean ± SD ($n = 10$). $^{*}p < 0.05$ compared to nonirradiated control group. $^{\#}p < 0.05$ compared to 448 nm group. $^{\S}p < 0.05$. SD, standard deviation.

Statistical analysis

One-way analysis of variance (IBM® SPSS® Statistics 22) was used with Tukey's test as a *post hoc* test to compare differences among the groups, for all statistical analyses. A p value of <0.05 was considered significant.

Results

Temperature elevation of P. gingivalis *bacterial suspension after UV irradiations*

The temperature elevations of *P. gingivalis* bacterial suspensions after UV irradiations were very low, under 0.5°C, for all wavelengths with a significant difference compared with control ($p < 0.05$), except for 448 nm. The shortest UV wavelength 265 nm produced the highest temperature elevation (Fig. 2).

CFUs of UV-irradiated bacteria

No bacterial colonies were observed in the groups receiving 265 or 285 nm irradiation. Approximately 1 log reduction of CFUs were observed in the 310 nm irradiated group compared with the control group ($p < 0.05$; 1.02 log reduction for *P. gingivalis*, 0.74 for *P. intermedia*, 1.27 for *F. nucleatum*, 1.59 for *A. actinomycetemcomitans*, and 1.26 for *S. oralis*). The number of CFUs for both 365 and 448 nm did not differ from those of controls for any bacteria (Fig. 3).

P. gingivalis *growth (OD changes) following UV irradiation*

P. gingivalis irradiated at 265 or 285 nm showed little growth during 24 h, while a slight growth increase was

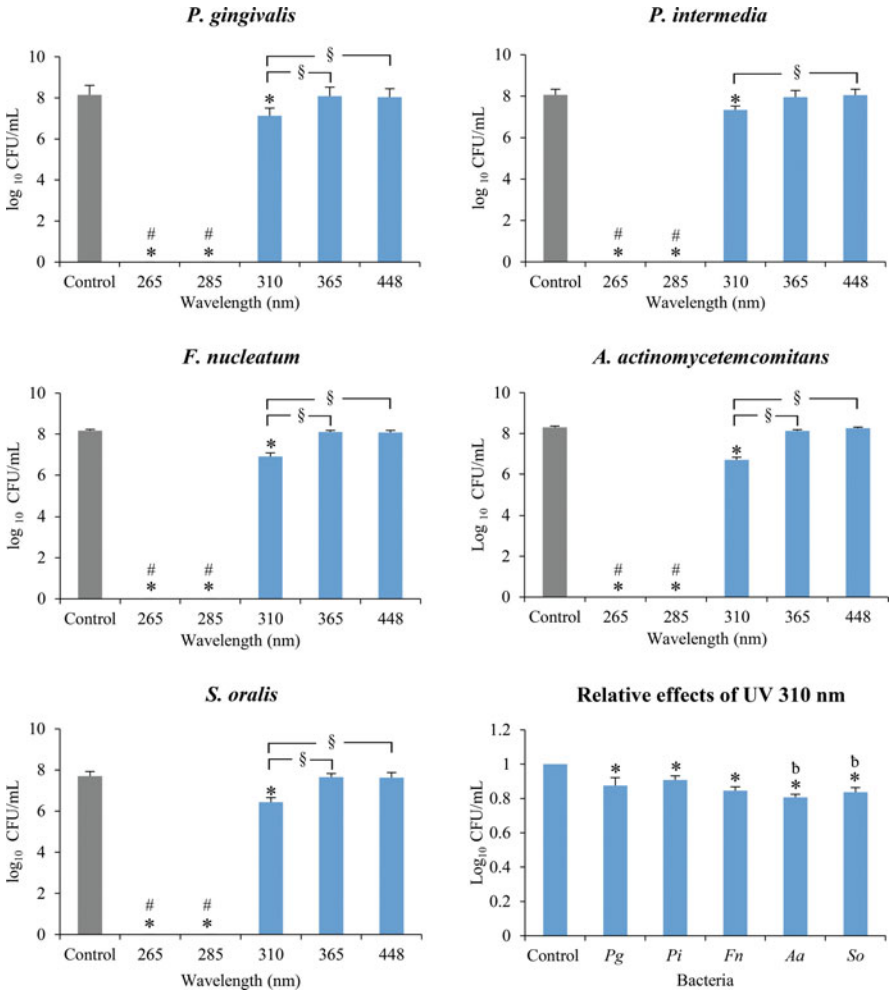

FIG. 3. CFUs following UV LED irradiations at various wavelengths on periodontopathic and oral bacterial suspensions. Data are presented as the mean ± SD ($n = 4$). $^{*}p < 0.05$ compared to nonirradiated control group. $^{\#}p < 0.05$ compared to 310, 365, and 448 nm groups. $^{b}p < 0.05$ compared to *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* groups. $^{\S}p < 0.05$. CFUs, colony forming units; SD, standard deviation.

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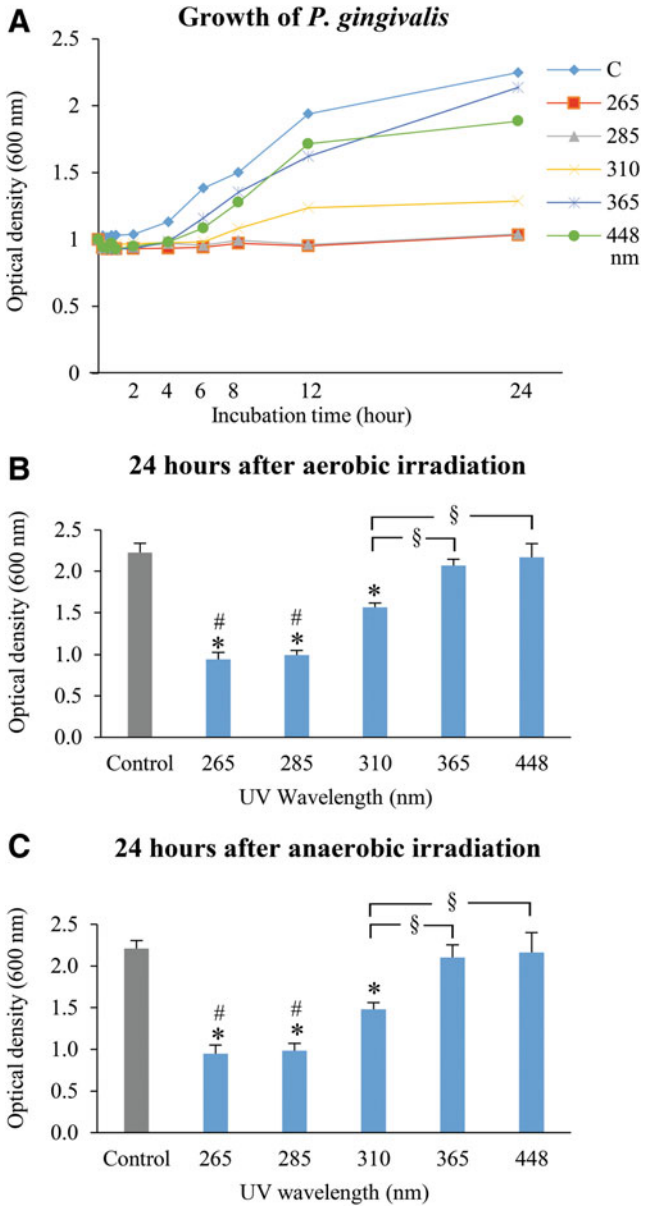

FIG. 4. *Porphyromonas gingivalis* growth in culture medium. Bacterial growth was indicated as an optical density (OD_{600}). **(A)** *P. gingivalis* growth curves after anaerobic UV LED irradiations, C: control. **(B)** *P. gingivalis* growth at 24 h after aerobic irradiation. **(C)** *P. gingivalis* growth at 24 h after anaerobic irradiation. Data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.05$ compared to nonirradiated control group. $^{\#}p < 0.05$ compared to 310, 365, and 448 nm groups. $^{\S}p < 0.05$. SD, standard deviation.

observed with 310 nm irradiation. *P. gingivalis* showed no difference in growth among 365 nm, 448 nm, and control groups (Fig. 4A). For irradiations under aerobic and anaerobic conditions, *P. gingivalis* growth showed similar growth trends (Fig. 4B, C).

SEM analysis of UV-irradiated P. gingivalis

There were no morphological changes of *P. gingivalis* in all UV-treated groups after 30 min or 1 day following irradiation. Morphological alterations with irregular and deformed cells were observed from day 2 postirradiation for 265 and 285 nm, and from day 3 for 310 nm. Boundaries of adjacent bacterial cells became blurred at that time. Five days after irradiation, *P. gingivalis* cells have no trace of their original forms and appeared completely broken and fragmented for 265, 285, and largely broken for 310 nm groups, while there were no notable changes of bacterial cell forms in 365 and 448 nm groups for a period of 5 days postirradiation (Fig. 5).

RNA quality and gene expression profiling in P. gingivalis *following 310 nm irradiation*

RIN value showed little change (around 8.0–9.1) up to 4 h following irradiation. However, the RIN value decreased substantially to under 2.5 on days 1, 2, 3, and 5.

At 30–60 min, gene expressions related to chaperones [*htp*G (PGN_0041), *clp*B (PGN_1208), *GroES* (PGN_1451), and *GroEL* (PGN_1452)] were remarkably increased and then gradually decreased. Whereas, SOS response-related genes [*UmuD* (PGN_1126), *UmuC* (PGN_1127), *RecA* (PGN_1057), and DNA polymerase III (PGN_1786)], and genes related to chromosome replication (PGN_0001), and cell division (PGN_0043), were inhibited more than twofold at 30-min postirradiation followed by a gradual recovery (Fig. 6).

Cytotoxicity of UV irradiations on HGF-1

Approximately 60 s irradiation at 265 or 285 nm completely devitalized HGF-1 on days 1 and 3. Also, 20 s treatment of H_2O_2 or BKC showed complete devitalization. 310 nm reduced viability by ~30% and 50% at days 1 and 3, respectively, whereas 365 and 448 nm resulted in no significant reduction compared with control group (Fig. 7).

Discussion

In this study, the bacteria were exposed to a total ED of ~600 mJ/cm^2 with a portable UV LED device *in vitro*. Temperature elevations of the bacterial solutions after irradiation were negligible at all wavelengths, and there should be no noteworthy influence from thermal effects induced by UV LED irradiation on bacterial viability. This nonthermal devitalization of bacteria may be one of the advantages of using UV LEDs, for bacterial reduction during periodontal treatment. It does not produce thermal damage to the surrounding periodontal tissues. This is in contrast to thermal devitalization/evaporation of bacteria using relatively high power lasers, such as CO_2, Nd:YAG, Diode, and Er:YAG lasers, which results in thermal damage to host tissues and cells.

Interestingly, with decreasing wavelength, the temperature-elevation increased proportionally, suggesting greater bacterial absorption of shorter wavelengths that may be related to the increased bactericidal effects of shorter wavelengths such as 265, 285, and 310 nm. In fact, a previous study showed that among UV LED wavelengths, UVC light ~254 nm is most readily absorbed by DNA, resulting in the strongest bactericidal effects.[29] Consequently, 1-week postirradiation, 265 and 285 nm completely suppressed CFU formation and 310 nm produced a significant 1 log reduction

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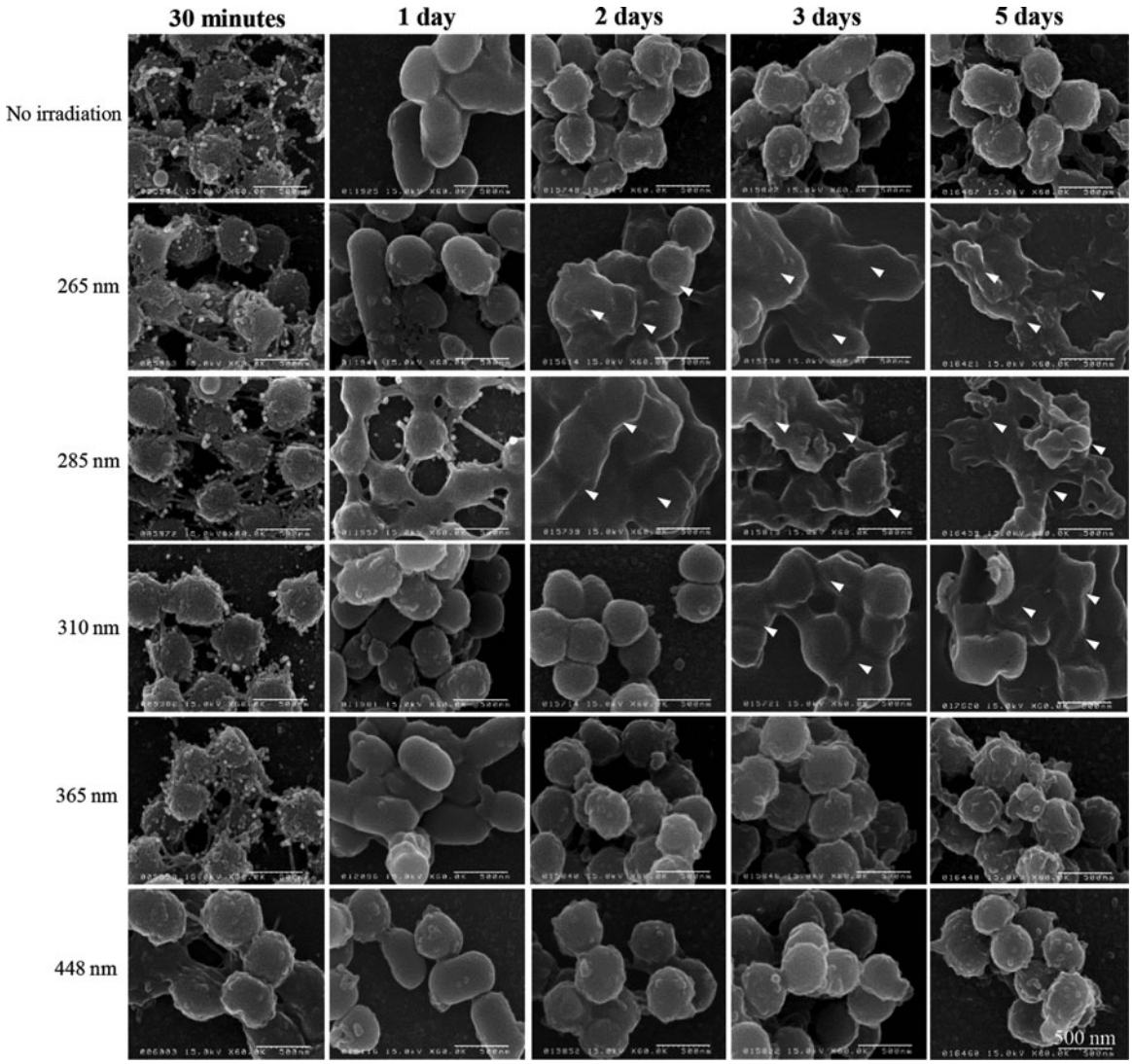

FIG. 5. Scanning electron microscope micrographs of *Porphyromonas gingivalis* following UV LED irradiation at various wavelengths. White arrowhead: alteration of bacterial cell morphology.

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(90% killing) of CFUs for all bacteria examined. Thus, 265 and 285 nm irradiations revealed strong and complete bactericidal effects, whereas 310 nm showed weaker bactericidal and growth suppression effects on bacteria.

P. gingivalis, following 265 and 285 nm irradiations, did not grow up to 24 h (no OD increase or decrease during that period). This was in accord with SEM findings of no destruction of *P. gingivalis* on day 1 for 265 and 285 nm. Further, following 310 nm irradiation, OD increased, to a lesser extent, indicating partial growth suppression. Interestingly, results were similar under both aerobic and anaerobic irradiation conditions, suggesting no influence by the presence of oxygen during irradiation.

With SEM analysis, initial alteration of bacterial cell morphology was observed around day 2 for 265 and 285 nm, and around day 3 for 310 nm. Severe destruction was observed on day 5 for these wavelengths. At the same time, according to the RIN value measurement, the total RNA from *P. gingivalis* irradiated at 310 nm showed no destruction until 4 h; however, the RNA severely degraded on days 1–5. Thus, it appears that UV wavelengths may not immediately devitalize bacteria by directly affecting bacterial cell walls. Previous studies described that shorter wavelength UVC irradiation can induce DNA pyrimidine dimer lesions that in turn inhibit cell proliferation and apoptosis, eventually leading to cell death.[30,31]

In contrast, a-PDT, which produces reactive oxygen species, shows a different mechanism to destroy bacteria. We have previously demonstrated that a-PDT with combined rose Bengal and blue LEDs immediately reduced OD reaching a minimal plateau after 40 min, suggesting the induction of immediate destruction of cells. Almost complete degradation of RNA has also been observed 3 h post-treatment.[32]

Bacteria must survive challenging conditions to colonize or invade host cells. These stresses can cause cellular proteins to denature and form insoluble aggregates that are devoid of biological activity.[33] Bacterial cells produce stress proteins including molecular chaperones. They help prevent, or manage, the misfolding and aggregation of proteins that

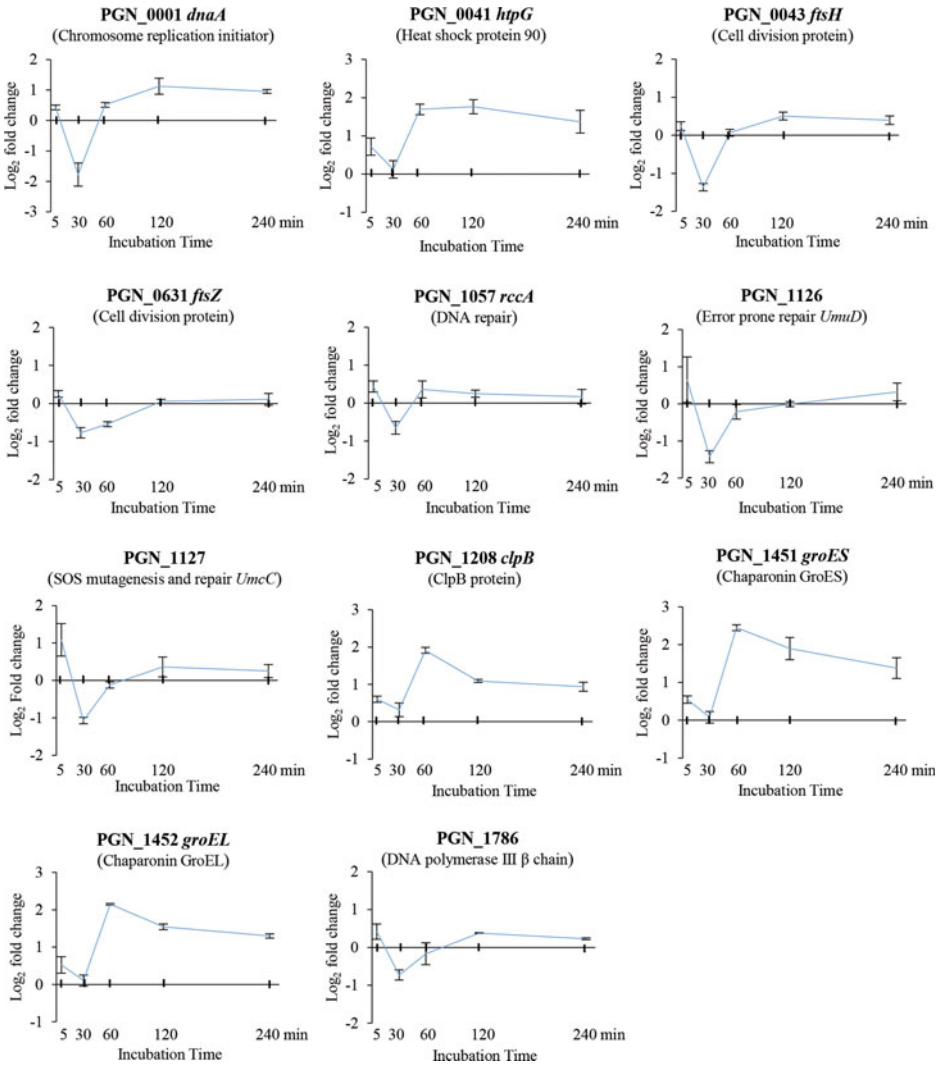

FIG. 6. Gene expression profiling of *Porphyromonas gingivalis* irradiated at 310 nm compared to nonirradiated control. The fold change [$\log_2$ fold change (vs. control)] in gene expression at each time point was calculated based on the comparative CT method. Data are presented as the mean ± SD ($n = 4$). SD, standard deviation.

are threats to all living organisms. In this study, following irradiation, chaperone genes (*GroEL*, *GroES*, *HtpG*, and *ClpB*) were rapidly upregulated after 30 min. This suggests that 310 nm UV irradiation may induce a broad stress response in *P. gingivalis*.[34,35] In addition, the bacteria surviving following irradiation (~10%) may have suffered nonfatal DNA damage affecting genes associated with metabolism and bacterial cell growth. Their chaperon gene expression levels remained relatively high after 60 min, suggesting that chaperone proteins may be providing support to damaged proteins.

SOS response in bacteria is a global response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced.[36] Most mutagenesis resulting from exposure to UV radiation requires the operation of a specialized system involving the *UmuD*, *UmuC*, *RecA*, and DNA polymerase III proteins, which allows translesion synthesis to occur on damaged DNA templates.[37] In our study, SOS response genes showed similar gene expression profilings, following a rapid decrease from 5 to 30 min and then a restoration to the control level, suggesting that 310 nm UV irradiation may inhibit gene expressions and induce a decrease in cell number at stationary phase. That is also supported by findings of similar expression profiles among DNA replication initiator (*DnaA*) and cell division protein (*FtsH* and *FtsZ*). In our previous study, blue LED (425–500 nm) did not degrade RNA but inhibited *P. gingivalis* growth by suppressing DNA replication and cell division-related genes.[27]

According to SEM and RNA findings with 310 nm irradiation, it took a relatively short time for the UV-irradiated bacterial cells to change gene expressions and a relatively long time to change cell morphology. This suggests that the most UV-irradiated bacteria may undergo chromosome inactivation, transcriptional suppressions, and production of defective proteins at least within 4 h. Then, the bacterial cell may begin to loose activity within 24 h. However, even after devitalization, cell structure will not be destroyed for up to 48 h. Some surviving bacterial cells may avoid the mutation of genes indispensable to life support, and instead, only transiently experience cell stress and growth suppression.

Finally, to evaluate phototoxicity of UV wavelengths to periodontal tissue, the effects on experimentally prepared HGF-1 cells were examined, as compared to a nonirradiated control and oral antiseptic positive controls (BKC and H_2O_2). Interestingly, the following irradiation effects on HGF-1 relative cell viability correlated with the irradiation effects on bacterial cell death. Both 265 and 285 nm

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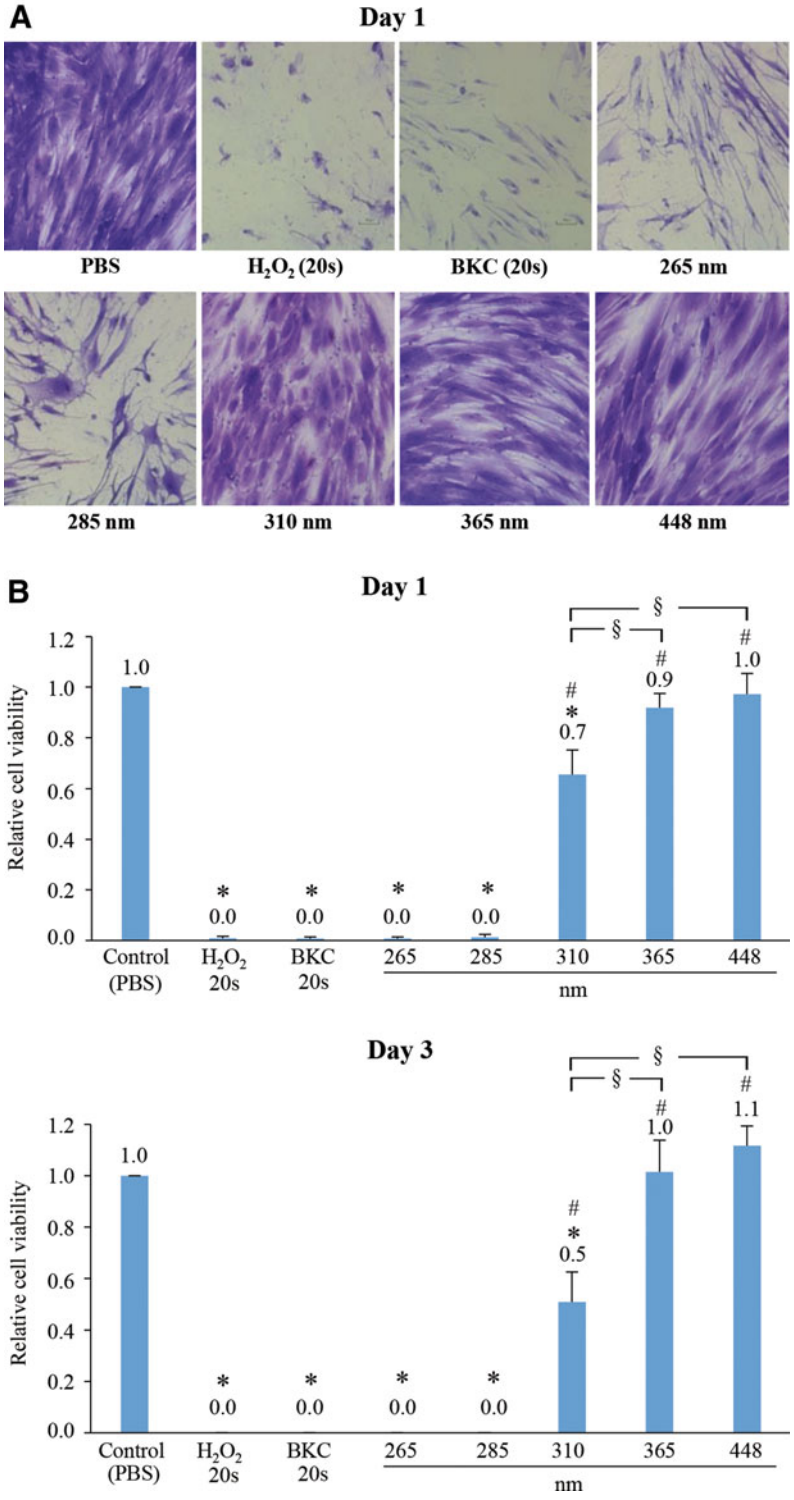

FIG. 7. **(A)** Crystal violet staining images of human gingival fibroblasts at day 1 after UV LED irradiation. **(B)** Viability of human gingival fibroblasts at days 1 and 3 after UV LED irradiations. Oral antiseptic agents [3% H_2O_2 (20 s) and 0.025% BKC (20 s)] were used as cell-damaging controls. Data are presented as the mean ± SD ($n = 3$). $^{*}p < 0.05$ compared to nonirradiated control group. $^{\#}p < 0.05$ compared to H_2O_2, BKC, 265, and 285 nm groups. $^{\S}p < 0.05$. BKC, benzalkonium chloride; H_2O_2, hydrogen peroxide; PBS, phosphate-buffered saline; SD, standard deviation; UV LED, ultraviolet light-emitting diode.

irradiations, and treatment with oral antiseptic agents (BKC and H_2O_2) for 20 s, severely damaged and devitalized the fibroblasts. In contrast, 310 nm irradiation showed much less harmful effects on the cells.

Since 310 nm irradiation has already been used to treat skin diseases in medicine, our findings with regard to the reduced phototoxicity of 310 nm irradiation support the possibility that it might also be effective for bacterial growth inhibition in periodontal therapy while posing less risk to the surrounding tissues. Even though 265 and 285 nm UV LED lights are clearly cytotoxic to human host cells, their strong bactericidal effects may be useful for disinfection of surgically exposed/isolated tooth or implant surfaces. While these results obtained from *in vitro* study do not necessarily ensure efficacy in clinical situations, we consider that UVC and UVB LED irradiations have the possibility for use as new means of periodontal and/or peri-implant therapy. We plan to pursue further studies regarding clinical applications.

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Conclusions

Under present irradiation conditions, both 265 and 285 nm may induce complete bactericidal effects, as well as complete death of irradiated gingival fibroblasts, *in vitro*; whereas 310 nm achieves partial killing and inhibition of bacterial growth with much less phototoxicity to fibroblasts. This suggests that UVC and UVB light with shorter wavelengths may have potential to be applied for bacterial suppression in periodontal and peri-implant therapy. Such UV light use may have different applications in various clinical situations depending on the wavelength.

Acknowledgments

We thank Dr. Kenji Matsushita, National Center for Geriatrics and Gerontology, and Drs. Shizuko Ichinose, Yuriko Sakamaki, Yujin Ohsugi, and Yutaro Kitanaka, TMDU for their advice and help. Support was provided by the Research Funding for Longevity Sciences (29-3) program administered by the National Center for Geriatrics and Gerontology.

Author Disclosure Statement

No competing financial interests exist.

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Received: June 20, 2018
Accepted after revision: November 22, 2018
Published online: April 5, 2019.

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Photodiagnosis and Photodynamic Therapy 32 (2020) 102033

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

The effect of antimicrobial photodynamic therapy using yellow-green LED and rose bengal on *Porphyromonas gingivalis*

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ARTICLE INFO

Keywords:
Antimicrobial photodynamic therapy
LED
Rose bengal
Porphyromonas gingivalis
Periodontal disease

ABSTRACT

Introduction: This study aimed to investigate the effects of a new antimicrobial photodynamic therapy (aPDT) system using yellow-green light-emitting diode (YGL) and rose bengal (RB) on *Porphyromonas gingivalis* (*Pg*) *in vitro*.

Materials and methods: Pg suspension mixed with RB was irradiated with YGL (565 nm) or blue light-emitting diode (BL, 470 nm) at 428 mW/cm^2 in comparison with chlorhexidine (CHG) treatment. The cells were cultured anaerobically on agar plates, and the number of colony-forming units (CFU) was determined. The treated suspension was anaerobically incubated, and the cell density (OD_{600nm}) was monitored for 24 h. Also, the viability of treated human gingival fibroblast (HGF-1) was measured using WST-8 assay. *Pg* morphology was observed with a scanning electron microscope. The RNA integrity number of aPDT-treated *Pg* was determined and gene expressions were evaluated by quantitative real-time polymerase chain reaction.

Results: RB + YGL (aPDT) demonstrated a significantly higher reduction of CFU, compared to RB + BL (aPDT) and CHG, furthermore the OD value rapidly decreased. Morphological changes of *Pg* with RB + YGL were more severe than with CHG. Although RB + YGL reduced HGF-1 viability, aPDT's impact was significantly lower than CHG's. With RB + YGL treatment, RIN values decreased; furthermore, gene expressions associated with DNA replication and cell division were remarkably decreased after 12 h.

Conclusion: The results of this study demonstrated that a novel aPDT system using RB + YGL may have potential as a new technical modality for bacterial elimination in periodontal therapy.

1. Introduction

Periodontal disease, a chronic inflammatory disease prevalent in both developed and developing countries, affects around 800 million people with the global burden of periodontal disease having increased by 26.6 % from 2007 to 2017 [1]. The primary etiologic factor of periodontitis is bacterial plaque [2], and *Porphyromonas gingivalis* (*Pg*), a black-pigmented anaerobic gram-negative bacterium, is one of the primary pathogens in periodontitis. *Pg* possesses many virulence factors (such as fimbriae, protease, hemagglutinins, and capsular polysaccharide) that allow it to contribute to disease [3,4]. The aim of periodontal treatment is to control periodontal infection and arrest the progression of the disease. Conventional treatment approaches including mechanical debridement and pharmacological therapy effectively improve the clinical periodontal condition; however, it is difficult to sufficiently eradicate pathogenic bacteria by these methods. Moreover, pharmacological therapy using antibiotics has many disadvantages, such as allergy, difficulty in maintaining therapeutic

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https://doi.org/10.1016/j.pdpdt.2020.102033
Received 30 July 2020; Received in revised form 16 September 2020; Accepted 25 September 2020
Available online 2 October 2020
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concentrations in periodontal pockets, and emergence of drug-resistant bacterial strains [5,6].

Light energy, delivered in such forms as lasers and light-emitting diodes (LEDs), has been applied for the management of periodontal diseases in recent years; furthermore, the effect of ultraviolet (UV) LEDs on periodontopathic bacteria is now studied *in vitro* [7,8]. In particular, antimicrobial photodynamic therapy (aPDT) is considered to be an alternative or auxiliary to the mechanical removal of infectious substances [9]. aPDT consists of three components; a nontoxic photosensitizer, harmless light, and oxygen [10,11]. By irradiating the photosensitizer with light of a specific wavelength, the photosensitizer is activated and reactive oxygen that is highly toxic to bacteria is produced. Resistant bacteria do not arise because aPDT has neither genotoxic nor mutagenic effects; consequently it is considered a safer choice for long-term treatment [12]. aPDT can destruct biofilm [13], and can impair the virulence of periodontal pathogens as well as detoxify bacterial endotoxins such as lipopolysaccharide.

Within the field of periodontal therapy, aPDT employing a red diode laser and blue dye agents, such as methylene blue or toluidine blue O, has been studied and clinically applied. On the other hand, we have previously examined the combination of a red dye such as rose bengal (RB) and blue light; these two being commonly used as a dental plaque disclosing agent and dental curing light, respectively. Thus, we initially focused on aPDT using this combination of RB and blue LED (BL) and have shown that aPDT using a combination of RB and BL effectively suppresses *Pg* colony formation [14,15]. However, BL's wavelength (470 nm) does not match the maximum absorption wavelength of RB. In fact, the absorption intensity of RB at 470 nm of BL is very low (approximately 0.1 absorbance unit [AU]) [16]. Therefore, a higher bactericidal effect could be expected with better-matched light sources. Importantly, the wavelength which is maximally absorbed by RB is approximately 550 nm [17,18]. To the best of our knowledge, no studies have reported the effects of aPDT using a wavelength approximating 550 nm.

Thus, the aim of this *in-vitro* study was to investigate the antimicrobial effects of red dye agent RB in combination with yellow-green LED (YGL) (565 nm: 0.6 AU) [16] with its wavelength close to the maximum absorption of RB (550 nm).

2. Materials and methods

2.1. Light sources

In this study, the following devices were used in tandem as a non-laser light source: a high power LED Controller (DC2200; Thorlabs Inc., Newton, NJ), used with either a mounted YGL [Lime (yellow-green); M565L3; Thorlabs Inc., Newton, NJ; wavelength 565 nm] or a mounted BL [Blue; M470L4; Thorlabs Inc., Newton, NJ; wavelength 470 nm]. The YGL or BL was placed under the 96 well plate (Falcon®, Corning Inc., Corning, NY) and the irradiation was performed at 428 mW/cm^2 from the bottom of each well (6.8 mm in diameter) under aerobic conditions. The energy output was measured above the well using a power meter (PM100D; Thorlabs Inc., Newton, NJ).

2.2. Preparation of the bacterial suspension

Pg ATCC 33277 was cultured into 9 mL of brain heart infusion medium (supplemented with 5 mg/L of hemin and 50 μg/L of vitamin K_1) and cultured anaerobically at 37℃ to the mid-log phase. Immediately before the experiment, the bacterial suspension was diluted, and the concentration was adjusted to 1.0×10^8 cells/mL using a counting chamber. After centrifugation of *Pg* suspension, the supernatant was removed, and the same volume of phosphate buffer saline (PBS) was added for resuspension.

2.3. Preparation of the photosensitizer and other solutions

RB (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was used as a photosensitizer. RB solutions were freshly prepared by mixing RB powder with PBS to obtain original concentrations of 2.5, 5, and 10 μg/mL (the final RB concentrations after being mixed with bacterial suspension were 0.4, 0.8, and 1.6 μg/mL, respectively). In RB and aPDT treatment groups, the mixed solution was allowed to stand for 1 min to permit the uptake of dye by bacteria before irradiation or culture.

As a positive control, 20 % chlorhexidine gluconate (CHG) solution (Wako Pure Chemical Industries, Osaka, Japan) was diluted with sterile distilled water to obtain original solutions of 1.2 % yielding final concentrations of 0.2 % after mixing with the bacterial suspension. After 20 s of exposure to CHG, its action was arrested by using sterile saline solution including a chlorhexidine inhibitor (0.75 g of L-alpha-lecithin dissolved in 3 mL of Tween 80, with the final volume adjusted by adding 97 mL of saline solution) instead of saline only solution during diluting procedure for cultivation [19].

2.4. Antimicrobial effects of RB + BL (aPDT) versus RB + YGL (aPDT) on Pg

Eight groups were employed in this experiment: 2 LED irradiation only groups (*Pg* with PBS; BL or YGL); six aPDT groups (*Pg* with RB, final concentrations 0.4, 0.8, and 1.6 μg/mL; BL or YGL). In each group, 100 μL of *Pg* suspension was placed in a well of a sterile 96-well flat-bottom plate. One minute after combining *Pg* with 20 μL of RB or PBS, the wells were exposed to BL or YGL for 20 s (8.56 J/cm^2). Finally, treated bacterial suspensions were serially diluted with saline. The suspensions were plated in triplicate onto Brucella agar plates (supplemented with 50 mL/L of horse blood, 50 mg/L of hemin and 50 mg/L of vitamin K1) incubated anaerobically at 37 °C for 1 week, and then colony-forming unit (CFU) were calculated.

2.5. Antimicrobial effects of RB + YGL (aPDT) versus CHG on Pg

Five groups were employed in this experiment: untreated control group (*Pg* with PBS); RB only group (*Pg* with RB, final concentration 1.6 μg/mL); YGL only group (*Pg* with PBS; YGL); aPDT group (*Pg* with RB; YGL); and positive control group (*Pg* with CHG, final concentration 0.2 %). In each group, 100 μL of bacterial suspension was placed in a well. One minute after combining *Pg* with PBS/RB/CHG, the wells were either exposed to no LED or 20 s YGL (8.56 J/cm^2). Finally, each group was incubated anaerobically for 1 week, and then CFUs were calculated.

2.6. Antimicrobial effects of RB + YGL (aPDT) on Pg at different irradiation times

Four groups were employed in this experiment: untreated control group (*Pg* with PBS); and aPDT groups (final concentration 1.6 μg/mL RB and YGL) with different irradiation times (0, 10, and 20 s). One minute after combining *Pg* with 20 μL PBS/RB, the wells were exposed to YGL for 0, 10, or 20 s (0, 4.28, and 8.56 J/cm^2). Finally, each group was incubated anaerobically, and then CFUs were calculated.

2.7. Cytotoxicity of RB + YGL (aPDT) on human gingival fibroblasts (HGF-1)

HGF-1 (ATCC CRL-2014, Manassas, VA) was cultured in Dulbecco's modified Eagle medium (Wako Pure Chemical Industries), supplemented with 10 % fetal bovine serum (FBS; Gibco, Carlsbad, CA), and 1 % antibiotic antimycotic mixture (Wako Pure Chemical Industries) in a humidified atmosphere with 5 % CO_2 at 37℃. HGF-1 cells were seeded in 96-well plates at 1.0×10^4 cells per well. 48 h after seeding, supernatants of HGF-1 cultures were removed and combined with PBS, 1.6 μg/mL RB, or 0.2 % CHG.

The five groups were treated as follows: one negative control group (HGF-1 with PBS for 1 min); CHG positive control group (HGF-1 with 0.2 % CHG for 20 s); RB group (HGF-1 with 1.6 μg/mL RB for 1 min) YGL group (HGF-1 with PBS for 1 min; YGL 20 s, 8.56 J/cm^2); RB + YGL (aPDT) group (HGF-1 with RB for 1 min; YGL 20 s, 8.56 J/cm^2). The HGF-1 viability was measured using WST-8 assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions on day 1 and 3 following treatment. The optical absorbance of samples was measured using fluorescence microplate reader at a wavelength of 450 nm (VMAX; Molecular Devices, Sunnyvale, CA). The ratio (%) of cell viability of irradiated or treated HGF-1 relative to that of untreated control was calculated.

2.8. Morphological analysis of Pg by scanning electron microscopy (SEM)

Cell morphology of *Pg* for the untreated control group (*Pg* with PBS), the CHG group (*Pg* with CHG for 20 s, final concentration 0.2 %), and the RB + YGL (aPDT) group (*Pg* with RB for 1 min, final concentration 1.6 μg/mL; YGL 20 s, 8.56 J/cm^2), was assessed by SEM. Prior to observations, samples were fixed using 2.5 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h. After washing three times in the same buffer, they were fixed in 1 % osmium tetroxide for 1 h and were dehydrated in a graded series of ethanol and dried in a critical point drying apparatus with liquid CO_2. The samples were spatter-coated with platinum and examined by scanning electron microscope (S-4500; Hitachi, Tokyo, Japan).

2.9. In-vitro Pg growth following RB + BL/YGL (aPDT)

Pg cell culture in the mid-log phase was centrifugated and adjusted with PBS to an OD_{600} value of 0.8. The *Pg* suspension (100 μL) was mixed with PBS/RB (20 μL) and six groups were prepared as follows: untreated negative control group (*Pg* with PBS); RB only group (*Pg* with RB, final concentration 1.6 μg/mL); BL only group (*Pg* with PBS; BL 20 s, 8.56 J/cm^2); YGL only group (*Pg* with PBS; YGL 20 s, 8.56 J/cm^2); RB + BL group (aPDT) (*Pg* with RB; BL 20 s); RB + YGL group (aPDT) (*Pg* with RB; YGL 20 s). After treatment, treated *Pg* was anaerobically incubated at 37°C and then OD_{600} was measured every 2 h from 2 to 24 h.

2.10. RNA quality and gene expression profiling of Pg following RB + YGL

Following RB (final concentration 1.6 μg/mL) + YGL (20 s, 8.56 J/cm^2) treatment, the *Pg* cell suspensions were mixed with RNAprotect™ Bacteria Reagent (Qiagen, Tokyo, Japan) to stabilize the bacterial RNAs. Total RNAs were prepared with the Trizol® Reagent (Invitrogen, Carlsbad, CA) and a PureLink® RNA Nano Kit (Invitrogen) according to the manufacturer's instructions. Bacterial disruptions were performed using a FastPrep® FP120 Instrument (Qbiogene, Carlsbad, CA). For the quantitative measurement of RNA degradation, the RNA integrity number (RIN) [20] was determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with RNA 6000 Nano LabChip® Kit (Agilent Technologies, Palo Alto, CA).

Reverse transcription was performed with random hexamers (Invitrogen) and Superscript™ II reverse transcriptase (Invitrogen). The design of the primers are shown in Table 1 based on our previous study [21]. Quantitative real-time PCR (qRT-PCR) was performed using a Thermal Cycler Dice® Real Time System III (Takara, Shiga, Japan) with SYBR Premix Ex Taq™ (Takara) as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. All data were analyzed based on the comparative threshold cycle (Ct) method [22]. The resulting relative values represented the relative expression level of a given gene compared with the level in the control at a corresponding incubation time point.

2.11. Statistical analysis

Data are presented as the mean ± standard deviation (SD). In Figs. 1, 3, 4 and 5, one-way analysis of variance (ANOVA) followed by Turkey's HSD post hoc test was performed to compare the differences among all the groups. In Fig. 2, two-way ANOVA with two unpaired factors were performed, and a simple main effect test was performed when the interaction had significances. Statistical procedures were performed using a PC software for Figs. 1, 2, 3, 4, and 5 (RStudio version 1.2.5001, Boston, MA).

3. Results

3.1. Antimicrobial effects of RB + BL versus RB + YGL (aPDT)

The effect of this newly proposed aPDT on *Pg* was tested by graded RB and YGL on *Pg* (Fig. 1). CFUs were reduced in an RB concentration-dependent manner (Fig. 1). YGL in combination with 0.4, 0.8, and 1.6 μg/mL of RB showed 1.1, 2.8, and 5.7 $\log_{10}$ reductions of CFUs, respectively. The highest antimicrobial effect, with a 5.7 $\log_{10}$ reduction, was observed at 1.6 μg/mL of RB ($p < 0.001$, compared to control). Comparing the effects of BL and YGL irradiation with the same concentration of RB, significantly higher antimicrobial effects were seen in

Table 1
PCR primers used in this study.

Gene name (Gene ID[a])	Direction[b]	Primer sequence (5′ – 3′)	Size (bp)	Description
dnaA (PGN_0001)	F	TTTGGAGGGCAATTTCGTAG	124	Chromosomal replication initiator
	R	TGTCACCGTACCGGGATATT		
dnaB (PGN_1378)	F	TGCCGATATGGTATGCTTCA	138	Replicative DNA helicase
	R	CGCAAACGTACATCATCCAC		
dnaE (PGN_0034)	F	GCAATCGTTTAGCCAAGCTC	132	DNA polymerase III subunit alpha
	R	GGGTATCGCGCATTACCTTA		
dnaG (PGN_1751)	F	TGTGCAGAAGTTCCAACTCG	136	DNA primase
	R	TAAGGCCTTGCTGTCTTCGT		
recA (PGN_1057)	F	GAATGGCCACGGAGAAGATA	137	DNA repair
	R	GTAACCGCCTACACCGAGAG		
polA (PGN_1771)	F	GAGACCGACTCGAAAGATGC	137	DNA polymerase I
	R	AGCGGACGCAAGAGATCTAA		
ftsH (PGN_0043)	F	GTAGGAGCCTCTCGTGTTCG	132	Cell division protein
	R	CTCATCATTGCCGGAGAAAT		
ftsZ (PGN_0631)	F	TACCACCGATCCGGAGTTAG	111	Cell division protein
	R	ACATTGCCAAGGTTGTCTCC		
sulA (PGN_1525)	F	CGCTGGGAGATCGTTACATT	101	Cell division inhibitor
	R	GGGGGAAAGACCTTTGAATC		

[a] Locus number from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NC_010729.1).
[b] F, forward. R, reverse.

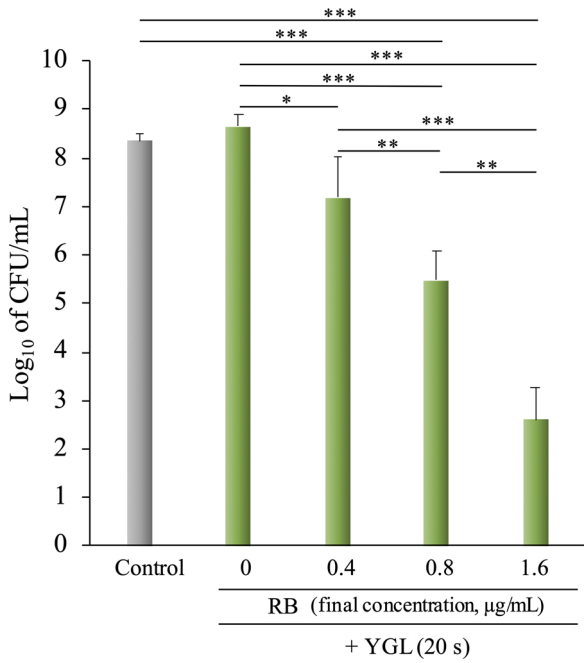

Fig. 1. Antimicrobial effects of RB + YGL (aPDT) on *Porphyromonas gingivalis* (*Pg*). *Pg* suspension mixed with/without RB was irradiated with YGL. *Pg* suspension with neither dye nor irradiation was employed as control. Data are presented as the mean ± SD of four independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (Tukey's HSD test). aPDT, antimicrobial photodynamic therapy. CFU, colony-forming units. RB, rose bengal. YGL, yellow-green LED. s, second.

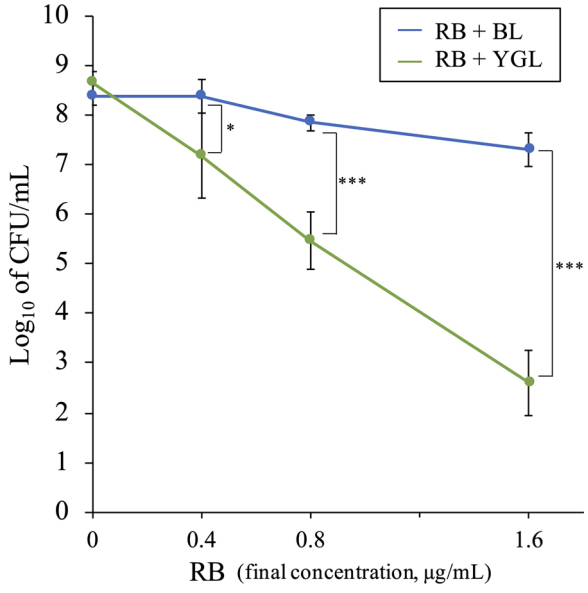

Fig. 2. Antimicrobial effects of RB + YGL/BL (aPDT) on *Porphyromonas gingivalis* (*Pg*). *Pg* suspension mixed with/without RB was irradiated with BL or YGL. Data are presented as the mean ± SD of four independent experiments. *: $p < 0.05$, ***: $p < 0.001$ (two-way ANOVA with two unpaired factors and a simple main effect test). aPDT, antimicrobial photodynamic therapy. CFU, colony-forming units. RB, rose bengal. BL, blue LED. YGL, yellow-green LED.

the RB + YGL group ($p < 0.05$ for 0.4 μg/mL, $p < 0.001$ for 0.8 and 1.6 μg/mL) and interaction was also detected between two groups (Fig. 2).

3.2. Antimicrobial effects of RB + YGL (aPDT) versus CHG on Pg

The antimicrobial effect of aPDT was compared to the 0.2 % CHG, which is commonly used as an oral antimicrobial agent. The CHG positive control showed a 2.5 $\log_{10}$ reduction of CFU compared to the untreated control group ($p < 0.001$). In contrast, 1.6 μg/mL of RB + 20 s YGL irradiation showed a significant 5.0 $\log_{10}$ CFU reduction compared to the untreated control ($p < 0.001$). Furthermore, RB + YGL showed a significant 3.0 $\log_{10}$ CFU reduction compared to the CHG ($p < 0.001$) (Fig. 3).

3.3. Antimicrobial effects of RB + YGL (aPDT) on Pg at different irradiation times

1.6 μg/mL RB with YGL irradiation showed reductions of approximately 5 $\log_{10}$ and 7 $\log_{10}$ at 10 and 20 s irradiation, respectively ($p < 0.001$ for both) (Fig. 4).

3.4. Cytotoxicity of RB + YGL (aPDT) on HGF-1

RB treatment reduced viability of HGF-1 to approximately 80 % and 95 % on day 1 and day 3. YGL irradiation reduced viability to approximately 50 % and 70 % at day 1 and 3 ($p < 0.001$ for both, compared to control). Cell viability after RB + YGL (aPDT) was reduced to approximately 30 % and 20 % at day 1 and day 3 ($p < 0.001$ for both) (Fig. 5). CHG treatment reduced cell viability to a much greater, as well as significant, extent; approximately 10 % and 5 % at day 1 and 3, respectively ($p < 0.05$ for both), compared to RB + YGL (Fig. 5).

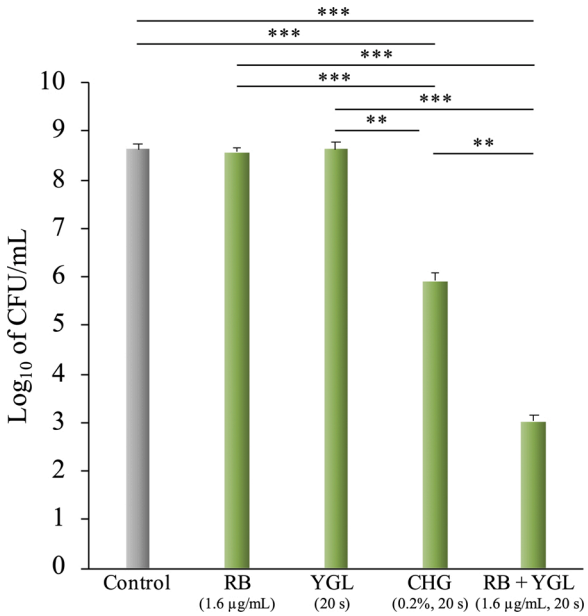

Fig. 3. Antimicrobial effects of RB + YGL (aPDT) versus CHG on *Porphyromonas gingivalis* (*Pg*). CHG was used as positive control as a bactericidal agent. *Pg* suspension was treated with RB, YGL, CHG or RB + YGL (aPDT). *Pg* suspension with neither dye nor irradiation was employed as control. Data are presented as the mean ± SD of four independent experiments. **: $p < 0.01$, ***: $p < 0.001$ (Tukey's HSD test). aPDT, antimicrobial photodynamic therapy. RB, rose bengal. YGL, yellow-green LED. CHG, 0.2 % chlorhexidine gluconate. CFU, colony-forming units. s, second.

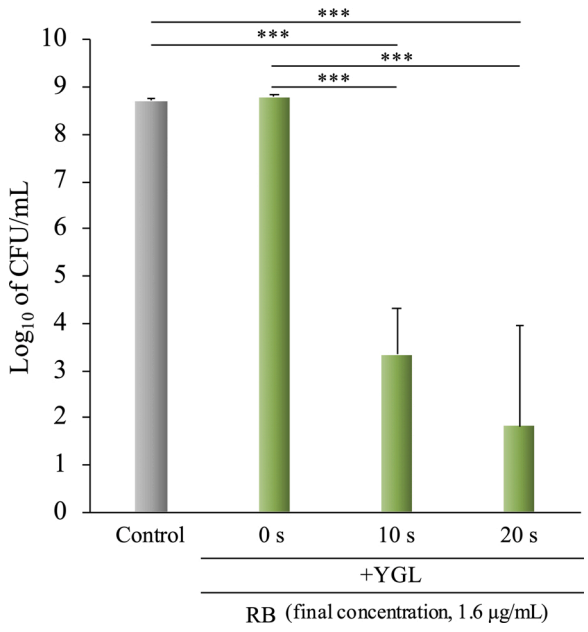

Fig. 4. Antimicrobial effects of RB + YGL (aPDT) on *Porphyromonas gingivalis* (*Pg*) at different irradiation times. *Pg* suspension was mixed with RB and irradiated (0, 10, 20 s). *Pg* suspension with neither dye nor irradiation was employed as control. Data are presented as the mean ± SD of four independent experiments. ***: $p < 0.001$ (Tukey's HSD test). aPDT, antimicrobial photodynamic therapy. RB, rose bengal. YGL, yellow-green LED. CFU, colony-forming units. s, second.

3.5. Morphological analysis of Pg by SEM

Pg cell surface morphology in the control group was relatively smooth. On the other hand, the *Pg* cell surface became rougher overtime for the CHG group. Likewise, *Pg* cell surfaces became rougher with aPDT, with more severe changes, such as injuring of cell-membrane/wall, observed after 3 and 12 h, compared to CHG (Fig. 6).

3.6. In-vitro Pg growth following aPDT using RB + BL/YGL

With RB, as with LEDs (YGL/BL), bacterial growth gradually increased and reached a plateau with no difference from untreated control after 8 h. In the RB + BL (aPDT) group, the growth was inhibited temporarily and then increased to a plateau (1.8). In contrast a dramatic reduction of growth rate in the RB + YGL (aPDT) group was observed until 6 h, finally reaching the lowest plateau of 0.1 OD_{600} (Fig. 7).

3.7. RNA quality and gene expression profiling of Pg following RB + YGL

The degradation of total RNAs was examined using non-irradiated samples (control) and RB + YGL (aPDT) samples. Based on the RIN, control samples showed similar RIN values (between 9.2 and 9.6) over time, whereas aPDT samples showed a gradual reduction of 9.4, 7.8, and 7.1 at 0, 3, and 12 h, respectively (Fig. 8). Gene expressions related to DNA replication (*dnaA*, *dnaB*, *dnaE*, *dnaG*, *recA*, and *polA*) increased their transcripts at 3 h but all decreased at 12 h. Gene expressions related to cell division (*ftsH* and *ftsZ*) showed similar expression patterns to DNA replication-related genes (Fig. 9). On the other hand, *sulA* increased at 3 h and the expression at 12 h was almost the same as control.

4. Discussion

Applications of aPDT are gradually being performed in the medical field. In the dental field, the main application has been as a combination of blue dye agent (such as methylene blue and toluidine blue O) and red

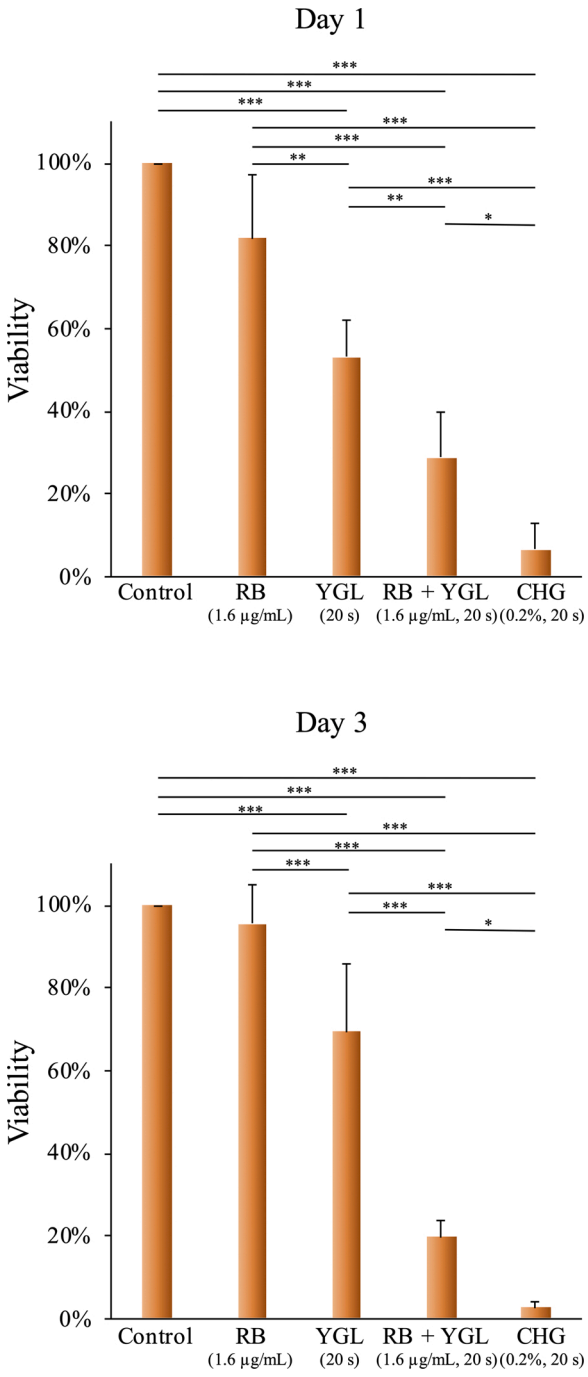

Fig. 5. Cytotoxicity of RB + YGL (aPDT) on human gingival fibroblasts (HGF-1). Viability of HGF-1 at day 1 and 3 was determined after RB, YGL, RB + YGL (aPDT), or CHG. HGF-1/PBS with neither dye nor irradiation was employed as control. Data are presented as the mean ± SD of five independent experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ (Tukey's HSD test). aPDT, antimicrobial photodynamic therapy. RB, rose bengal. YGL, yellow-green LED. CHG, 0.2 % chlorhexidine gluconate. s, second.

laser/LED. Zanin et al. [23,24] reported that a combination of red diode laser and Toluidine blue O significantly reduced bacterial viability after irradiation *in vitro*. Umeda et al. [25] reported that irradiation of red LED on methylene blue showed bactericidal effects on *Pg* and *Aggregatibacter actinomycetemcomitans*. On the other hand, different combinations of photosensitizers and LEDs have also been examined *in vitro*. Mahdi et al. [26] reported bactericidal effect on *Pg* and *Fusobacterium nucleatum* using a combination of curcumin/erythrosine and BL, and

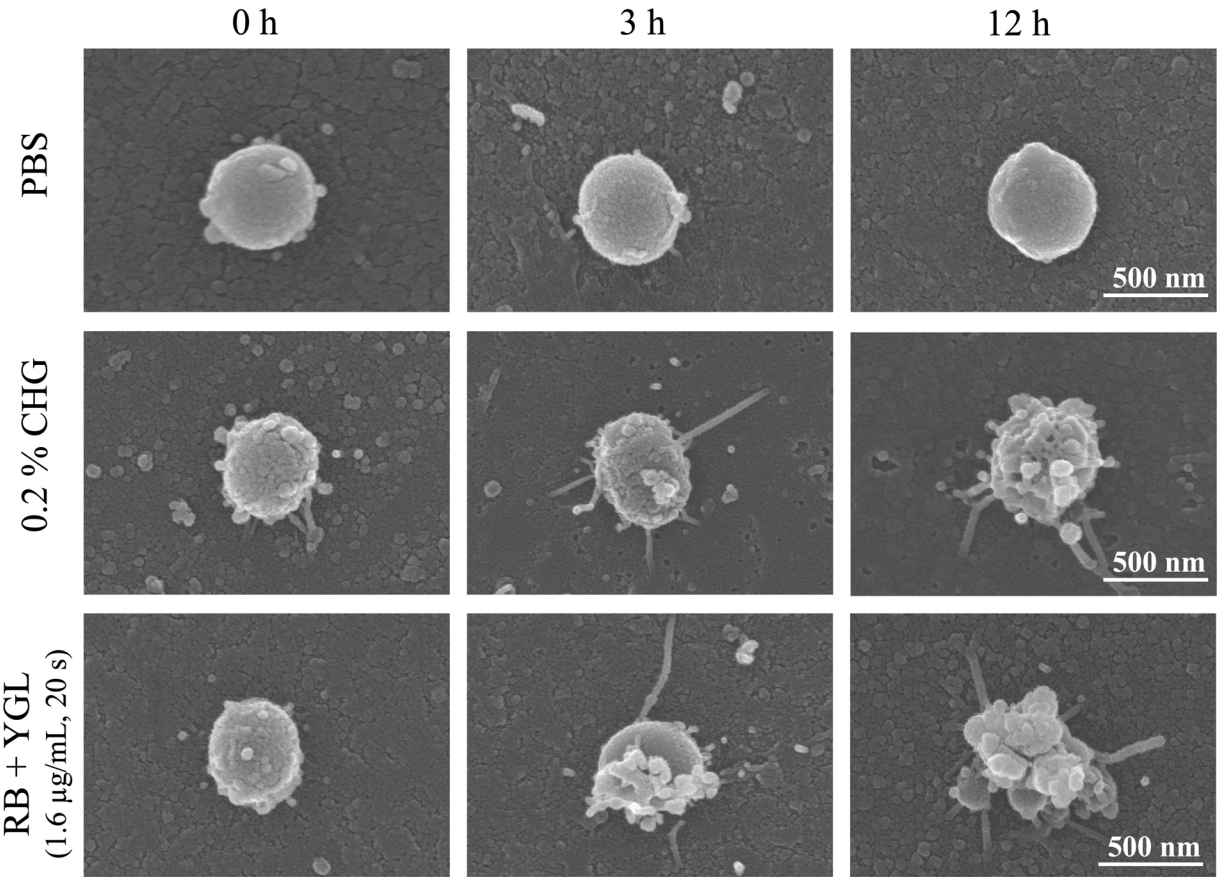

Fig. 6. SEM micrographs of *Porphyromonas gingivalis* after treatment with PBS (negative control), 0.2 % CHG (positive control) and RB + YGL (aPDT). SEM, Scanning electron microscope. aPDT, antimicrobial photodynamic therapy. YGL, yellow-green LED. RB, rose bengal. CHG, 0.2 % chlorhexidine gluconate. PBS, phosphate buffer saline. s, second. h, hour.

also Chui et al. [14] revealed significant bactericidal effect of RB and BL on *Pg*.

In performing aPDT, it is important to establish the optimum combination of light and photosensitizer in order to maximize the effectiveness of aPDT. The maximal effect of aPDT is exhibited only when the maximum absorption wavelength of the dye matches the wavelength of the light used. However, that has not always been the case in previous studies using RB. Regarding the application of RB with the highest absorption wavelength of 550 nm, several previous studies have used BLs (470 nm) and evaluated the effects of aPDT [15,21,27]. Recently, Durkee et al. [16] evaluated aPDT using RB and Green LED (525 nm) on *Pseudomonas aeruginosa in vitro*. *P. aeruginosa* was irradiated at 6 mW/cm^2 for 15 min and 95 % of bacterial growth inhibition was observed. Nonetheless, the low absorption by RB at 525 nm of Green LED (only 0.3 AU) is still a limitation [16].

To date, aPDT studies using an LED light with the wavelength closest to RB have not been conducted. The present study is the first investigation using YGL (565 nm), which is closest to the maximum absorption wavelength of RB. In our study, this new aPDT treatment employing RB + YGL significantly decreased viable bacteria as compared to RB + BL. This difference in antimicrobial effect may be a result of the compatibility of the light's wavelength with the present photosensitizer (RB). With aPDT using RB and YGL, the number of bacteria was significantly decreased in a dye concentration-dependent manner up to 1.6 μg/mL. Previously, Chui et al. [14] reported that a 6.3 $\log_{10}$ reduction was obtained after an aPDT treatment of 30 J/cm^2 BL (1.0 W/cm^2 and 30 s) and 1.6 μg/mL RB. In the present study, a similar 5.7 $\log_{10}$ reduction was observed with the novel aPDT consisting of the much lower energy level of 8.36 J/cm^2 YGL (428 mW/cm^2 and 20 s) and 1.6 μg/mL RB.

The antimicrobial effect of this new aPDT was also compared to 0.2 % CHG which is used as a conventional bacterial disinfect in medicine, especially for oral products. Results showed that RB + YGL had greater antimicrobial effect on *Pg*, furthermore, SEM also revealed more severe *Pg* damage with RB + YGL as compared to CHG. Regarding the effects on HGF-1 cells which were examined in order to evaluate the cytotoxicity of the RB + YGL; RB alone as well as YGL alone showed some toxicity to HGF-1, and RB + YGL showed more toxicity to HGF-1. However, compared to CHG which severely damaged and almost devitalized the fibroblasts, the toxicity of RB + YGL was significantly lower. These results suggested that RB + YGL possesses higher antimicrobial activity but less harmful effect compared to CHG.

Bacterial growth was rapidly inhibited by RB + YGL. OD value reached the lowest plateau (OD_{600nm} = 0.1) at 6 h after treatment and gradually decreased further until 12 h, suggesting that the destruction of bacterial cells had progressed rapidly. To understand the bacterial growth-inhibitory mechanism of RB + YGL, RNA was collected from the treated bacterial suspension, and its degradation was assessed by the RIN values [20]. Results demonstrated that bacterial RNA was gradually degraded from 0 to 12 h by RB + YGL treatment, but, although reduced, the RIN value still remained at 7.1 at 12 h after treatment, suggesting that all bacteria were not completely destroyed at 12 h. Looking at gene expressions of the surviving bacteria, the gene expressions related to DNA replication and cell division (*dnaA*, *dnaB*, *dnaE*, *dnaG*, *recA*, *polA*, *ftsH*, and *ftsZ*) increased at 3 h, but at 12 h they decreased remarkably compared to non-treated control. These results indicate that the ability for growth was almost lost even in surviving *Pg*, 12 h after aPDT treatment. Interestingly, gene expression of *sulA* increased at 3 h and remained present at 12 h. *sulA* is reportedly induced by the DNA damage response and serves to delay the cell cycle and inhibit cell division until

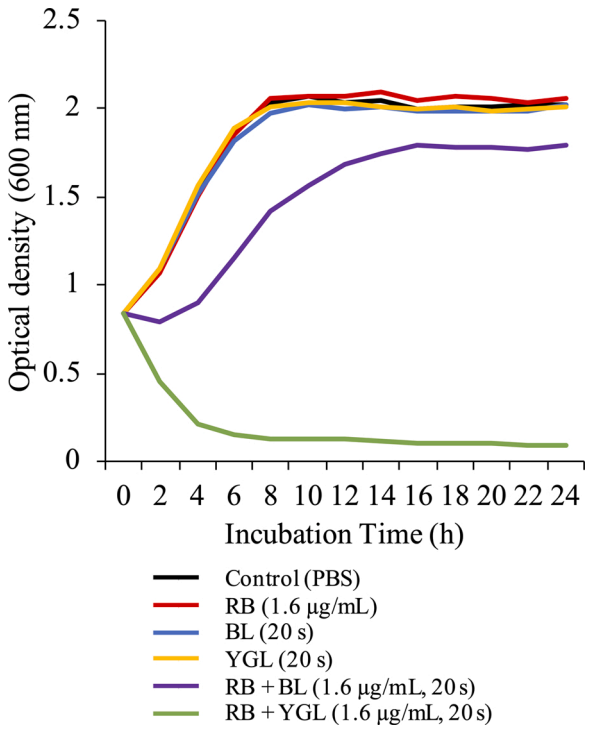

Fig. 7. *In-vitro Porphyromonas gingivalis* (*Pg*) growth following RB + BL/RB + YGL (aPDT). *Pg* cells (initial $OD_{600nm} = 0.8$) were treated with PBS, RB, BL/YGL, or aPDT (RB + BL/RB + YGL) and incubated anaerobically. OD was measured every 2 h for 24 h after treatment. aPDT, antimicrobial photodynamic therapy. RB, rose bengal. BL, blue LED. YGL, yellow-green LED. OD, optical density. s, second. h, hour.

the DNA is repaired [28]. Thus, the remaining expression of *sulA* is not contradictory to the loss of growth activity. In addition, the SEM images showed progressive cell destruction, becoming remarkable at 12 h. These results suggested that this novel aPDT system induced fatal bacterial damage even with the short-term irradiation, indicating that the treated bacteria could not survive for long.

In the present study, gene expressions in *Pg* following aPDT treatment were clarified. Interestingly, light-irradiation itself can alter the gene expression of *Pg* cells. Chui et al. [21] reported that 470 nm BL irradiation has led to remarkably decreased expression of genes associated with chromosomal DNA replication and cell division. Also, Aung et al. [7] demonstrated that the 310 nm ultraviolet LED irradiation has transiently suppressed the transcripts of SOS response- and cell division-related genes. Thus, an antibacterial effect by aPDT may be partly due to changes in various bacterial gene expressions induced by light itself. For further understanding of antibacterial mechanisms of aPDT, it seems necessary to investigate the characteristics and influence of lights at various wavelengths as well.

In addition to its bactericidal effect, aPDT has been expected to promote periodontal healing, due to its biomodulatory effects, by inhibiting host-derived cytokines and inflammatory cytokines. Braham et al. [29] demonstrated that aPDT using methylene blue and 670 nm diode laser has inactivated tumor necrosis factor-α and interleukins-1β from human endothelial cells. Also, Huang et al. [30] and Tanaka et al. [31] reported that aPDT using red diode laser and methylene blue promotes cell chemotaxis, local vasodilation and collagen synthesis, and angiogenesis in the treatment of arthritis (*in-vivo* study).

In recent years, aPDT has been applied occasionally for periodontal treatment in daily practice. In patients with chronic periodontitis, the adjunctive use of aPDT with scaling and root planing (SRP) has shown improvement in clinical periodontal parameters compared with those of SRP alone, although the beneficial effects have been short term [32]. A recent randomized controlled study of Monzavi et al. [33] showed that SRP plus aPDT (using 810 nm diode laser with indocyanine green) led to complete resolution of inflammation and significant reduction of pocket depth, even though other parameters were the same as SRP alone. Also, de Oliveira et al. [34] compared the effects of SRP and aPDT (660 nm diode laser with phenothiazine chloride) for the treatment of aggressive periodontitis. They showed that similar clinical improvements in gingival index, bleeding of probing, pocket depth, and clinical attachment level were observed in both treatment groups. Chambrone et al. [35] concluded in a review that aPDT provides similar clinical improvements in pocket depth and clinical attachment level when compared with conventional periodontal therapy for both periodontitis

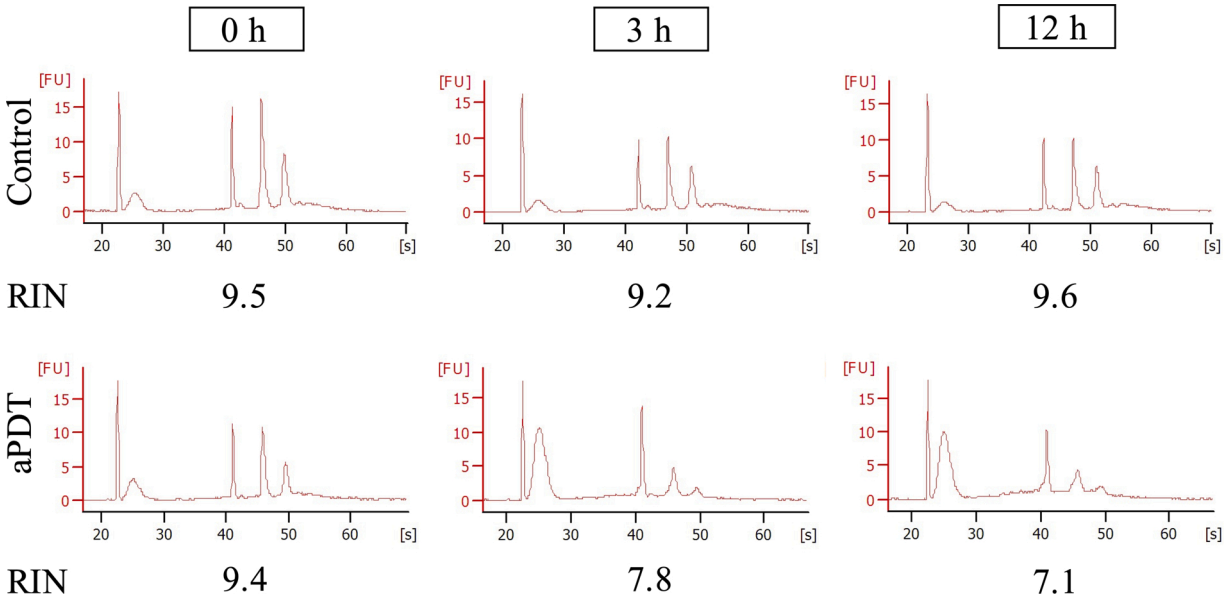

Fig. 8. RNA quality of *Porphyromonas gingivalis* (*Pg*) following RB + YGL. After purification of *Pg* total RNA from the non-treated control and RB + YGL (aPDT), the RNA quality was examined by using an Agilent 2100 bioanalyzer. The RNA integrity number (RIN) indicates the extent of RNA degradation. The RIN values (10: intact – 1: totally degraded) are shown below each graph image. aPDT, antimicrobial photodynamic therapy. RB, rose bengal. YGL, yellow-green LED. RIN, RNA integrity number. FU, fluorescence. s, second. h, hour.

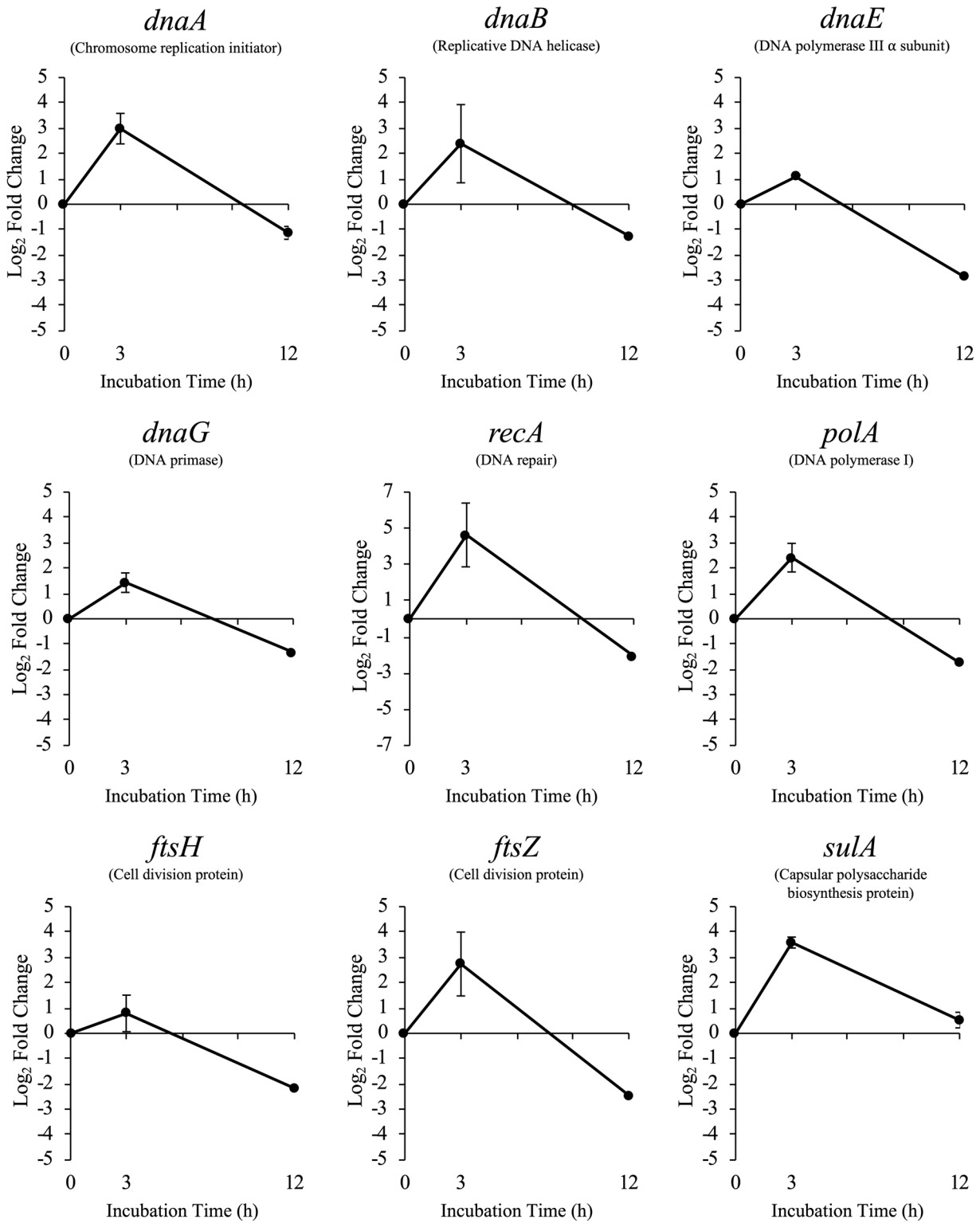

Fig. 9. Gene expression profiling of *Porphyromonas gingivalis* after RB + YGL (aPDT). The fold change [$\log_2$ fold change (vs. control)] in gene expression at each time point was calculated based on the comparative CT method. aPDT, antimicrobial photodynamic therapy. RB, rose bengal. YGL, yellow-green LED. h, hour.

and peri-implantitis patients in the short term. Thus, the positive effects of existing aPDT systems are still limited to the short term, suggesting that the antimicrobial effect with conventional aPDT systems may be clinically weak. Therefore, the present novel RB plus YGL aPDT system with its strong antimicrobial effect might be more effective than conventional aPDT systems, although further detailed basic and clinical studies are necessary to ensure the safety and effectiveness of this new system.

5. Conclusions

Within the limits of this study, aPDT using a combination of RB and YGL demonstrated significantly higher antimicrobial effects on *Pg* with less cytotoxicity as compared to CHG *in vitro*. These results suggest this

novel aPDT may have potential as a new therapeutic modality for bacterial elimination in periodontal therapy.

Declaration of Competing Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (JSPS KAKENHI 16K11825 to A.A., 16K11842 to K.H., and 17K11981 to Y.T.). We would like to express our special thanks to Dr. Noriko Kuwahara at Nihon University School of Dentistry at Matsudo, as well as to Drs. Ayako Mimata, Masako Akiyama, and Yoshiyuki Sasaki at Tokyo Medical and Dental University for their kind advice and help with this study.

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Photodiagnosis and Photodynamic Therapy 44 (2023) 103860

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Effects of various light-emitting diode wavelengths on periodontopathic bacteria and gingival fibroblasts: An *in vitro* study

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ARTICLE INFO

Keywords:
Light-emitting diode
Porphyromonas gingivalis
Gingival fibroblasts
Periodontal disease
Antibacterial effects

ABSTRACT

Background: In recent years, light has been used for bacterial control of periodontal diseases. This *in vitro* study evaluated the effects of light-emitting diode (LED) irradiation at different wavelengths on both *Porphyromonas gingivalis* and human gingival fibroblasts (HGF-1).

Methods: P. gingivalis suspension was irradiated with LEDs of 365, 405, 450, 470, 565, and 625 nm at 50, 100, 150, and 200 mW/cm^2 for 3 min (radiant exposure: 9, 18, 27, 36 J/cm^2, respectively). Treated samples were anaerobically cultured on agar plates, and the number of colony-forming units (CFUs) was determined. Reactive oxygen species (ROS) levels were measured after LED irradiation. The viability and damage of HGF-1 were measured through WST-8 and lactate dehydrogenase assays, respectively. Gene expression in *P. gingivalis* was evaluated through quantitative polymerase chain reaction.

Results: The greatest reduction in *P. gingivalis* CFUs was observed on irradiation at 365 nm with 150 mW/cm^2 for 3 min (27 J/cm^2), followed by 450 and 470 nm under the same conditions. While 365-nm irradiation significantly decreased the viability of HGF-1 cells, the cytotoxic effects of 450- and 470-nm irradiation were comparatively low and not significant. Further, 450-nm irradiation indicated increased ROS production and downregulated the genes related to gingipain and fimbriae. The 565- and 625-nm wavelength groups exhibited no antibacterial effects; rather, they significantly activated HGF-1 proliferation.

Conclusions: The 450- and 470-nm blue LEDs showed high antibacterial activity with low cytotoxicity to host cells, suggesting promising bacterial control in periodontal therapy. Additionally, blue LEDs may attenuate the pathogenesis of *P. gingivalis*.

1. Introduction

Periodontal disease is an inflammatory condition characterized by destruction of tooth-supporting tissues. Although the disease progression can be influenced by various environmental elements, as well as host systemic conditions, bacterial plaque is considered the primary etiological factor [1], and plaque control through reinforcement of oral hygiene instructions and professional mechanical plaque removal is performed as the first step in the management of periodontal disease. However, conventional periodontal therapies cannot completely eradicate bacteria from periodontal pockets and root surfaces with complex morphology [2,3]. To reinforce the elimination of bacteria, antibiotics are accordingly administered as an adjunct to mechanical plaque debridement; however, they also have disadvantages, such as allergy, presence/emergence of drug-resistant bacteria, and difficulty in maintaining optimal drug concentrations in periodontal pockets [4].

Recently, the application of light energy has been identified as a new therapeutic method for controlling bacterial infections in periodontal diseases [5–7]. Light-emitting diode (LED) is a non-coherent light, and has advantages over lasers, such as the ability to irradiate a large area

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https://doi.org/10.1016/j.pdpdt.2023.103860
Received 31 July 2023; Received in revised form 23 October 2023; Accepted 23 October 2023
Available online 24 October 2023
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with less invasiveness, lower cost, and smaller size of the equipment. These characteristics suggest that phototherapy using LED may be useful for periodontal care in both professional and home settings. Several studies have used LEDs as the light source for antimicrobial photodynamic therapy (aPDT) to inactivate pathogenic bacteria and suppress periodontal inflammation [8]. Photodynamic reactions of aPDT occur upon light irradiation of photosensitizers in the presence of oxygen [9], resulting in the production of reactive oxygen species (ROS) that damage diverse bacterial structures, thereby inducing an antibacterial effect. Moreover, a specific wavelength of LED light is known to suppress some periodontopathic bacteria by irradiation alone in the absence of exogenous photosensitizers [10–13]. However, till date, only few studies have compared the antibacterial effects of various wavelengths of LED light. The experimental conditions across these previous studies were not uniform, and a precise comparison of their results is difficult. Moreover, some wavelengths of light can activate host cells and, conversely, they may also promote bacterial growth [14,15]. Thus, it is necessary to investigate the appropriate conditions of irradiation time and power of LEDs, as well as the selection of wavelengths for the application of LEDs alone in clinical practice.

In the present *in vitro* study, we evaluated the effects of various LED light wavelengths on *Porphyromonas gingivalis*, a major periodontal pathogen associated with periodontitis, and gingival fibroblasts under the same irradiation conditions.

2. Materials and methods

2.1. Light sources

A high-power LED controller and mounted LEDs of various wavelengths were used (DC2200; Thorlabs Inc., Newton, NJ, USA): ultraviolet A light (UVA; peak wavelength: 365 nm), blue-violet light (405 nm), blue light (450 and 470 nm), yellow-green light (565 nm), and red light (625 nm). During irradiation, the irradiances were measured above the well using a power meter (PM100D; Thorlabs Inc.). The irradiations were performed one well at a time, from the bottom of each well (6.8 mm in diameter), with irradiances of 50, 100, 150, and 200 mW/cm^2 for 3 min (radiant exposure: 9, 18, 27, and 36 J/cm^2, respectively).

2.2. Preparation of bacterial suspension

P. gingivalis (ATCC 33277) obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) was maintained on a trypticase soy blood agar plate [16] under anaerobic conditions. A loopful (1.0 mm in diameter) of bacteria was taken from the plate and anaerobically cultured in 9 mL of brain heart infusion medium (Difco Laboratories, Franklin Lakes, NJ, USA) supplemented with 5 mg/L of hemin (Fujifilm Wako Pure Chemical Industries, Osaka, Japan) and 1 mg/L of vitamin K1 (KOA ISEI Co., Ltd., Yamagata, Japan) at 37°C. Immediately before the experiment, the bacterial suspension in the mid-log phase was diluted to adjust the concentration to 1×10^8 cells/mL using a counting chamber. After centrifugation of the bacterial suspension, the supernatant was removed and replaced with the same volume of 0.9% w/v sodium chloride (NaCl) solution for resuspension.

2.3. Culture of human gingival fibroblasts (HGF-1)

HGF-1 (cell line ATCC CRL-4061) obtained from ATCC were cultured in Dulbecco's modified Eagle medium (Fujifilm Wako Pure Chemical Industries) supplemented with 10 % fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1 % antibiotic antimycotic mixture (Fujifilm Wako Pure Chemical Industries) in a humidified atmosphere with 5 % carbon dioxide at 37°C. HGF-1 were seeded at 1.0×10^4 cells per well in 96-well flat-bottom plates (Falcon®, Corning Inc., Corning, NY, USA). Forty-eight hours after seeding, the medium was removed, and 200 μL of 0.9% w/v NaCl was added.

2.4. Antibacterial effects of various wavelengths of LED on P. gingivalis

Two hundred microliters of *P. gingivalis* suspension was added to each well of a sterile 96-well flat-bottom plate. LED irradiation at different wavelengths (365, 405, 450, 470, 565, and 625 nm), with irradiances of 50, 100, 150, and 200 mW/cm^2, was performed for 3 min from the bottom of each well under aerobic conditions (radiant exposure: 9, 18, 27, and 36 J/cm^2, respectively). After 3 min of irradiation, the bacterial suspensions were serially diluted with 0.9% w/v NaCl solution and plated in triplicate onto trypticase soy blood agar plates. They were incubated anaerobically at 37 °C for 1 week, and the colony forming units (CFU) were calculated.

2.5. Viability of HGF-1 following LED irradiation with different wavelengths

HGF-1 were irradiated with different LED wavelengths (365, 405, 450, 470, 565, and 625 nm) at 27 J/cm^2 (150 mW/cm^2 for 3 min). The viability of HGF-1 was measured using the WST-8 (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) and lactate dehydrogenase (LDH) assays (Cytotoxicity Detection Kit, Roche, Mannheim, Germany) according to the manufacturer's instructions on Day 1 and Day 3 following treatment. The optical absorbance of the samples was measured using a fluorescence microplate reader at 450 and 490 nm (VMAX, Molecular Devices, Sunnyvale, CA, USA). The ratio of the cell viability of irradiated HGF-1 cells relative to that of the untreated control was calculated using the WST-8 assay. The relative cytotoxicity was expressed as the ratio of the LDH activity in irradiated cells to that in nonirradiated cells. The LDH assay for each sample dish was performed in duplicate, and the mean value was used for statistical analyses.

2.6. Measurement of total ROS on P. gingivalis

Intracellular ROS were detected using a commercially available kit (ROS Assay Kit-Highly Sensitive 2′, 7′dichlorofluorescin diacetate [DCFH-DA]; Dojindo), according to the manufacturer's instructions. A working solution of highly sensitive DCFH-DA dye was added to the *P. gingivalis* pellet to make a *P. gingivalis* suspension. Two hundred microliters of *P. gingivalis* suspension was added to each well of a sterile 96-well flat-bottom plate. The *P. gingivalis* suspension was irradiated with LEDs at different wavelengths (365, 450, and 625 nm) at 150 mW/cm^2 for 30 s, 1 min, or 3 min (radiant exposure: 4.5, 9, or 27 J/cm^2, respectively). After the irradiation, the *P. gingivalis* suspension was incubated anaerobically at 37 °C for 30 min. The suspensions were washed three times with sterile saline solution, and the changes in the relative fluorescence intensities of ROS at 485 and 520 nm were detected using a plate reader (FLUOstar® OPTIMA-6, BMG Labtech Japan Ltd., Saitama, Japan).

2.7. RNA quality and gene expression profiling of P. gingivalis following LED irradiation

The *P. gingivalis* suspension was irradiated with LEDs (450 and 625 nm) at 150 mW/cm^2 for 3 min (radiant exposure: 27 J/cm^2) and was then mixed with RNA protect™ Bacteria Reagent (Qiagen, Tokyo, Japan) to stabilize the bacterial RNAs. Bacteria were disrupted using a FastPrep® FP120 Instrument (QBiogene, Carlsbad, CA, USA), and total RNAs were isolated using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and a PureLink® RNA Nano Kit (Invitrogen), according to the manufacturer's instructions. For quantitative measurement of RNA degradation, the RNA integrity number [17] was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with an RNA 6000 Nano LabChip® Kit (Agilent Technologies). Reverse transcription was performed using random hexamers (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen). Quantitative polymerase chain reaction was performed using a Thermal Cycler Dice® Real

Time System III (Takara, Shiga, Japan) with SYBR Premix Ex Taq™ (Takara) as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. The primers used are listed in Table 1 [12,18,19]. All data were analyzed based on the comparative threshold cycle method [20].

2.8. Statistical analysis

Data are presented as mean ± standard deviation. Since a Shapiro-Wilk test indicated that the data were consistent with a normal distribution, one-way analysis of variance followed by Tukey's HSD post hoc test was performed to compare the bacterial count (Fig. 1 and 2), viability of HGF-1 (Fig. 3 and 4), ROS induction level (Fig. 5), and gene expression level (Fig. 6) in each group. All statistical analyses were performed using a statistical software program (RStudio version 1.2.5001; Boston, MA, USA).

3. Results

3.1. Antibacterial effects of LED on P. gingivalis at various wavelengths

The effect of LED irradiation on *P. gingivalis* was evaluated at different wavelengths (365, 405, 450, 470, 565, and 625 nm) (Fig. 1). The CFU counts following LED irradiation at 365, 405, 450, and 470 nm decreased with increasing irradiances, whereas the CFU counts in the 565- and 625-nm wavelength groups did not differ from those in the control, even at the highest irradiance (36 J/cm^2). Under the same irradiation conditions [27 J/cm^2 (150 mW/cm^2, 3 min)], the greatest reduction in CFUs compared to the untreated control was observed in the 365-nm (3.6 log reduction) wavelength group, followed by the 450-nm (2.2 log reduction) and 470-nm (1.8 log reduction) wavelength groups (Fig. 2).

3.2. Effects of LED irradiation with different wavelengths on HGF-1 viability

The viability of HGF-1 on Day 1 and Day 3 following LED irradiation at different wavelengths is shown in Fig. 3. On Day 1, 365-nm irradiation significantly reduced the viability of HGF-1 (81.5 %) compared to that in the control. Although a slight reduction in viability was also observed in the 450-nm and 470-nm wavelength groups (91.2 % and 91.6 %, respectively), the viability in these groups did not differ from that in the control. In contrast, an increase in viability was observed in the 565-nm and 625-nm wavelength groups (114.1 % and 109.1 %, respectively), although there was no significant difference compared with the viability in the control.

On Day 3, a significant reduction in viability was observed in the 365-nm and 405-nm wavelength groups (58.2 % and 72.8 %, respectively), and the 450- and 470-nm wavelength groups showed further reduction (87.9 % and 84.1 %, respectively); however, there was no significant difference compared with the viability in the control. The viability of the 565- and 625-nm wavelength groups had increased further (127.1 % and 132.5 %, respectively), with significant differences compared with that in the control. There was no significant difference in the LDH activity of the control and all irradiated groups on Day 1 and Day 3 (Fig. 4). The results suggest that the LEDs exerted an antibacterial

Table 1
PCR primers used in this study.

Gene name (Gene ID[a])	Direction[b]	Primer sequence (5′–3′)	Size (bp)	Description	Reference[c]
dnaA	F	TTTGGAGGGCAATTTCGTAG	124	Chromosome replication initiator	[12]
(PGN_0001)	R	TGTCACCGTACCGGGATATT			
dnaB	F	TGCCGATATGGTATGCTTCA	138	Replicative DNA helicase	[12]
(PGN_1378)	R	CGCAAACGTACATCATCCAC			
dnaG	F	TGTGCAGAAGTTCCAACTCG	136	DNA primase	[12]
(PGN_1751)	R	TAAGGCCTTGCTGTCTTCGT			
dnaE	F	GCAATCGTTTAGCCAAGCTC	132	DNA polymerase III α submit	[12]
(PGN_0034)	R	GGGTATCGCGCATTACCTTA			
recA	F	GAATGGCCACGGAGAAGATA	137	DNA repair	[12]
(PGN_1057)	R	GTAACCGCCTACACCGAGAG			
polA	F	GAGACCGACTCGAAAGATGC	137	DNA polymerase I	[12]
(PGN_1771)	R	AGCGGACGCAAGAGATCTAA			
htpG	F	AATGGAAAGACGGCAAGATG	91	Heat shock protein 90	[12]
(PGN_0041)	R	TTGAGGTCAGCAGGCTTTTT			
ftsH	F	GTAGGAGCCTCTCGTGTTCG	132	Cell division protein	[12]
(PGN_0043)	R	CTCATCATTGCCGGAGAAAT			
clpB	F	AGAAACTGCCCCATGTATCG	129	Component of the stress response protein	[12]
(PGN_1208)	R	CTAGCACGATGTGCTCCAAA			
hbp35	F	TCTTGCTTTCGTACACCATC	93	35 kDa hemin binding protein	
(PGN_0659)	R	GCTGTAGGCTCTGCTTTATC			
sulA	F	CGCTGGGAGATCGTTACATT	101	Capsular polysaccharide biosynthesis protein	[12]
(PGN_1525)	R	GGGGGAAAGACCTTTGAATC			
ftn	F	AGCACGCCTACGATATGA	100	Ferritin	
(PGN_0604)	R	TCCAATACAGAGCCGAACT			
rgpA	F	GCCGAGATTGTTCTTGAAGC	256	Arginine-specific cysteine proteinase RgpA	[19]
(PGN_1970)	R	AGGAGCAGCAATTGCAAAG			
rgpB	F	CCTACGTGTACGGACAGAGCTATA	70	Arginine-specific cysteine proteinase RgpB	[18]
(PGN_1466)	R	AGGATCGCTCAGCGTAGCATT			
kgp	F	AGCTGACAAAGGTGGAGACCAAAGG	186	Lysine-specific cysteine proteinase Kgp	[19]
(PGN_1728)	R	TGTGGCATGAGTTTTTCGGAACCGT			
fimA	F	CAGCAGGAAGCCATCAAATC	140	Structural subunit of the major fimbriae	[19]
(PGN_0180)	R	CAGTCAGTTCAGTTGTCAAT			
fimC	F	AATGAACCCGATGCCCTTAC	82	Minor component of fimbriae	
(PGN_0183)	R	TCACATCAGGAGCTTGCATATC			
fimD	F	AGCAACCACCTGTACGATATG	93	Minor component FimD	
(PGN_0184)	R	CACAGAGAGAGAGCCGTTTATC			

[a] Locus number from NCBI (http://www.ncbi.nlm.nih.gov/nuccore/NC_010729.1).
[b] F, forward. R, reverse.
[c] (blank), Original design in this study.

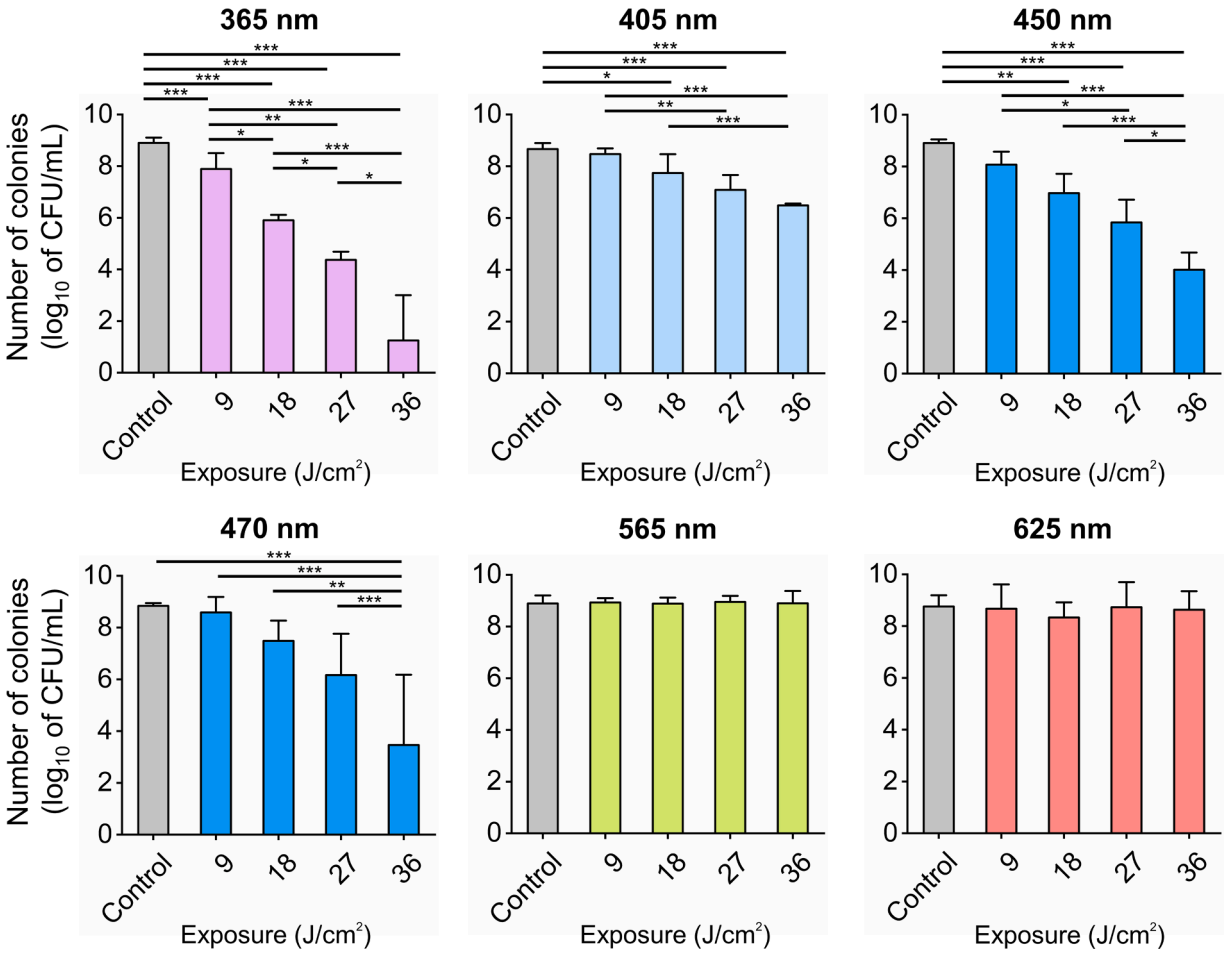

Fig. 1. Antibacterial effect of various wavelengths of LED on *Porphyromonas gingivalis*: comparison at different irradiances for each wavelength. *P. gingivalis* suspension was irradiated with 365-, 405-, 450-, 470-, 565-, and 625-nm LED. *P. gingivalis* suspension without irradiation was used as a control. Data are presented as the mean ± standard deviation of four independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Tukey's HSD test). CFU, colony-forming units.

effect mainly with short wavelengths (365, 450, and 470 nm). Together with the results of the viability test of HGF-1, we focused on blue LEDs (especially 450 nm) and investigated their antibacterial effects.

3.3. Total ROS induction in P. gingivalis following LED irradiation

The ROS levels of *P. gingivalis* were measured after irradiation with LEDs (365, 450, and 625 nm) at 4.5 J/cm^2 (150 mW/cm^2, 30 s), 9 J/cm^2 (150 mW/cm^2, 1 min), and 27 J/cm^2 (150 mW/cm^2, 3 min. At 4.5 J/cm^2 and 9 J/cm^2, the amount of ROS generated by the 450-nm group was greater than that of the 365- and 625-nm groups, and approximately 1.1–1.2 times higher than that of the control group, although there was no significant difference (data not shown). The amount of ROS generated with 450-nm LED at 27 J/cm^2 was the highest among the three groups and was 1.4 times that in the control. Further, the 365-nm LED induced a significantly higher ROS, which was 1.2 times higher than that in the control. The ROS level after 625-nm irradiation was similar to that in the control (Fig. 5).

3.4. RNA quality and gene expression profiling of P. gingivalis following LED irradiation

Gene expression of *P. gingivalis* following 450- and 625-nm irradiation was examined (Fig. 6). Irradiation with 450-nm wavelength decreased the expression of many genes, including genes related to chaperones (*htpG* [PGN_0041] and *clpB* [PGN_1208]), SOS response (*recA* [PGN_1057]), DNA replication (*polA* [PGN 1771]), *P. gingivalis* specific protease (gingipains) (*rgpB* [PGN_1466], *kgp* [PGN_1728], and *rgpA* [PGN_1970]), and structures on the bacterial surface (fimbriae) (*fimA* [PGN_0180], *fimC* [PGN_0183], and *fimD* [PGN_0184]); the expression of hemin binding protein (*hbp35* [PGN_0659]) reduced significantly compared with that in the control. Further, in the 625-nm wavelength group, expression of *recA* and *fimA* was significantly reduced, but genes related to cell division (*ftsH* [PGN_0043]), iron-binding protein [*ftn* (PGN 0604)], and *polA* were upregulated.

4. Discussion

In this study, we evaluated the effects of various LED irradiation wavelengths on *P. gingivalis* and HGF-1 under identical irradiation conditions. Several studies have shown the phototoxic effects of blue LED without exogenous photosensitizers on periodontal bacteria, including *P. gingivalis* [10,21–23]. We previously reported that ultraviolet and blue LEDs have antibacterial effects against *P. gingivalis* [9,12,13,24]. In the present study, irradiation with UVA (365 nm), blue-violet (405 nm), and blue (450 and 470 nm) LEDs reduced the number of *P. gingivalis* cells, which is consistent with previous reports. The antibacterial effect increased in an irradiance-dependent manner. Aung et al. reported the absence of any antimicrobial effect of UVA (365 nm) and blue (448 nm) LEDs at low irradiance levels (600 mJ/cm^2) against *P. gingivalis* [13]. The radiant exposure of LED light in this study was approximately four times higher than that used in their study, and we considered that the disparity in light exposure could be the primary reason for the antibacterial effect observed in our study. In other words,

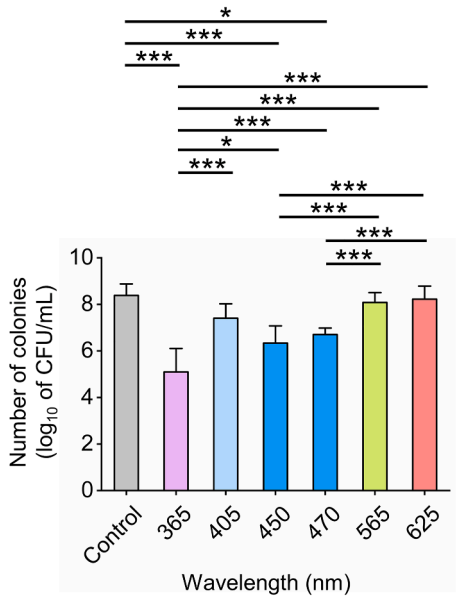

Fig. 2. Antibacterial effect of various wavelengths of LED on *Porphyromonas gingivalis*: comparison at the same irradiance among wavelengths. *P. gingivalis* suspension was irradiated with 365-, 405-, 450-, 470-, 565-, and 625-nm LED at 27 J/cm^2 (150 mW/cm^2, 3 min). *P. gingivalis* suspension that underwent no irradiation was employed as control. Data are presented as the mean ± standard deviation of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's HSD test). CFU, colony-forming units.

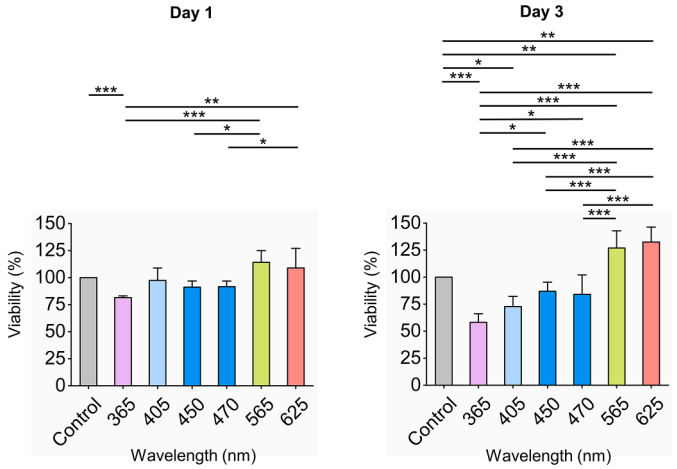

Fig. 3. Viability of human gingival fibroblasts (HGF-1) following LED irradiation with different wavelengths at the same irradiance. Relative proliferation of cells by WST-8 assay on Day 1 and Day 3 after irradiation [27 J/cm^2 (150 mW/cm^2, 3 min)] compared to the nonirradiated control cells. Data are presented as the mean ± standard deviation of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's HSD test).

a certain threshold of light energy would be required to induce an antibacterial effect. For the clinical application of LED irradiation in periodontal treatment, the high antibacterial effects must be accompanied with minimal damage to host cells. Although in our preliminary study, UVA and blue light exerted strong antibacterial effects after irradiation at 36 J/cm^2 (200 mW/cm^2, 3 min), high cytotoxicity on HGF-1 was also observed; HGF-1 viability in the 365-, 405-, and 450-nm wavelength groups on Day 3 was reduced to 50.3 %, 61.5 %, and 65.3 %, respectively. Following the results of preliminary study, we investigated the effects of LED irradiation on *P. gingivalis* and HGF-1 cells at 27 J/cm^2 (150 mW/cm^2, 3 min) with lower cytotoxicity.

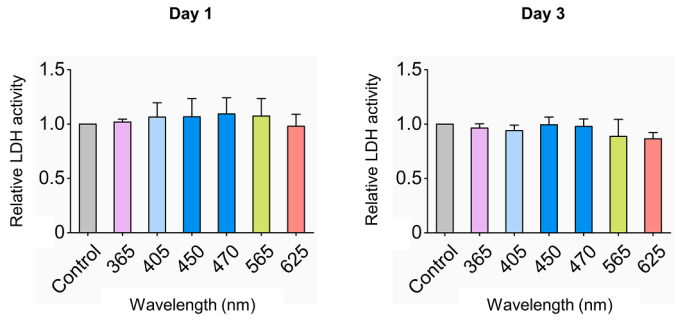

Fig. 4. Lactate dehydrogenase (LDH) activity of human gingival fibroblasts (HGF-1) following LED irradiation with different wavelengths at the same irradiance. Relative LDH activity in the cultured medium of HGF-1 on Day 1 and Day 3 after irradiation [27 J/cm^2 (150 mW/cm^2, 3 min)] compared to that of nonirradiated control cells. Data are presented as the mean ± standard deviation of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's HSD test).

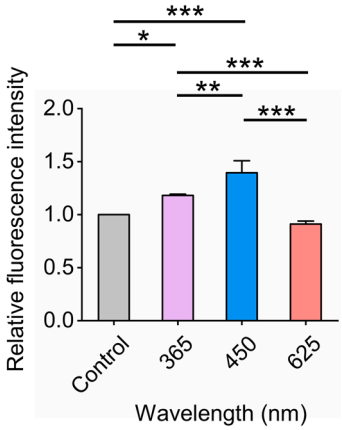

Fig. 5. Total reactive oxygen species (ROS) in *Porphyromonas gingivalis* following 365-, 450-, and 625-nm LED irradiation. Data are presented as the mean ± standard deviation of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Tukey's HSD test).

Iron is an essential nutrient for many species of bacteria to survive inside mammalian hosts. *P. gingivalis* degrades hemoglobin to derive heme, a complex of iron and the tetrapyrrole protoporphyrin IX [25], and endogenous porphyrin is produced when the bacterium further acquires iron from heme. Porphyrin as a photosensitizer in bacterial cells can absorb light and generate ROS, leading to disruption of the bacterial cell membrane and apoptosis [10,26,27]. Yoshida et al. have confirmed that the action of aPDT is mediated by endogenous protoporphyrin IX within *P. gingivalis* bacteria subsequent to irradiation with 460-nm LED light [27]. The present study also found a significant increase in ROS production after 450-nm light irradiation, suggesting that the antibacterial action of the blue light is related to ROS generation, which is associated with endogenous porphyrins in *P. gingivalis*. In contrast, the antibacterial effect and cytotoxicity of UVA (365 nm) were higher than those of blue light (450 and 470 nm). These results suggest that UVA exerts its effects not only through ROS production but also by other mechanisms, such as alteration of gene expression by photochemical reactions. However, it remains controversial whether UVA induces DNA damage and represses RNA synthesis like UVC (260 nm).

On the other hand, no antibacterial effects were observed with yellow-green (565 nm) or red (625 nm) LED irradiation. This is consistent with previous findings on the minimal antibacterial effects of a single application of yellow-green and red LED lights [28,29–31]. Although the radiant exposure used in this study (maximum 36 J/cm^2)

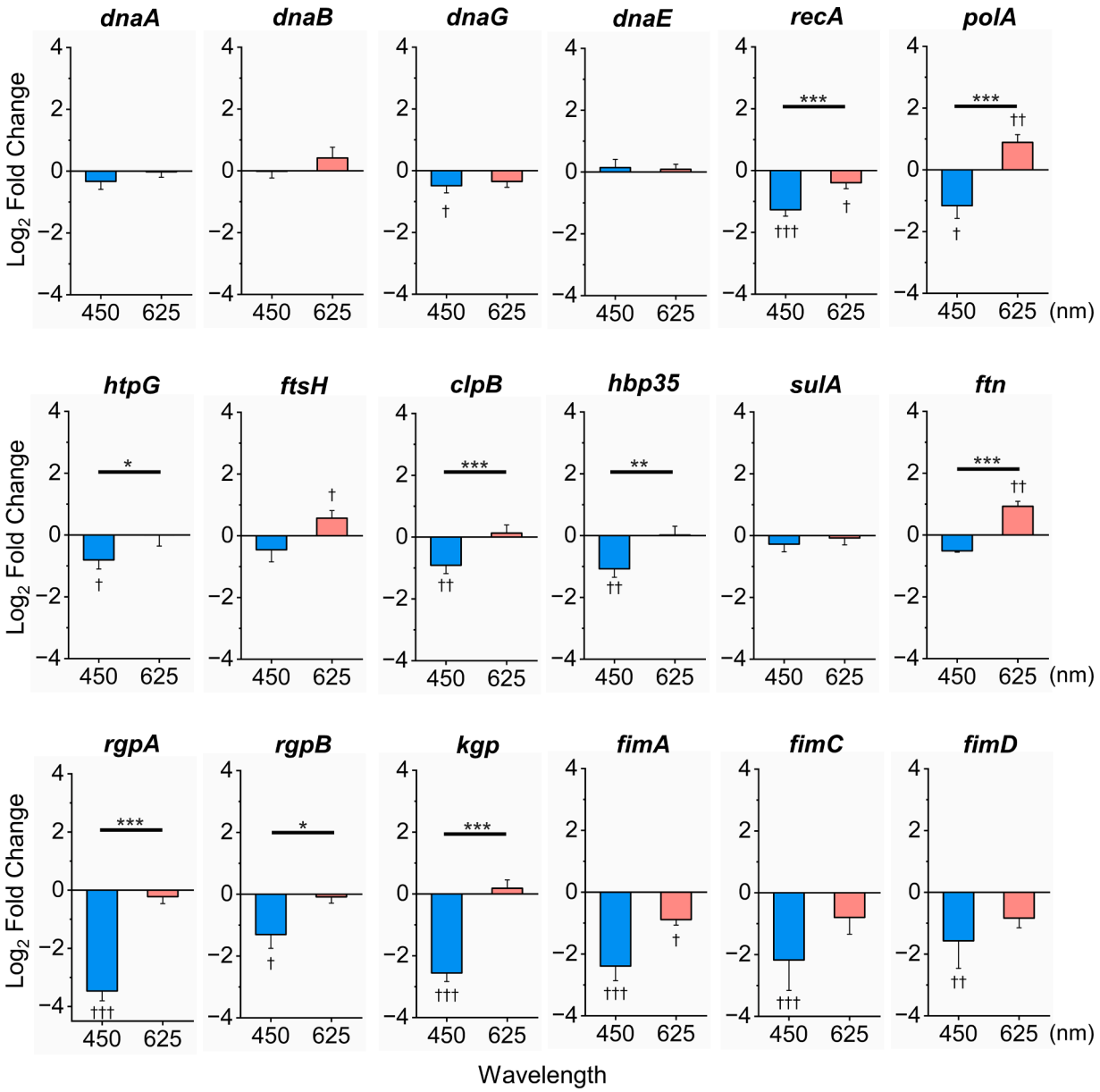

Fig. 6. Gene expression profiling of *Porphyromonas gingivalis* following 450- and 625-nm LED irradiation. The fold change [$\log_2$ fold change (*vs*. control)] in gene expression was calculated based on the comparative threshold cycle method. Data are presented as the mean ± standard deviation of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (450 nm *vs* 625 nm) $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ (*vs* control) (Tukey's HSD test).

was much higher than that used in previous studies by Kitanaka et al. (565 nm, 8.56 J/cm^2) [28] and Moslemi et al. (625 nm, 6 J/cm^2) [30], we still could not observe any antibacterial effect. Unlike the UVA and blue LEDs, red LED might promote the growth of *P. gingivalis*. Kim et al. reported that the viability of *P. gingivalis* increased after 8 h irradiance at 625 nm LED (172.8 J/cm^2) compared to the non-irradiated control [15]. *P. gingivalis* proliferation was not observed in the present study; however, HGF proliferation following 565- or 625-nm LED irradiation was observed. Vinck et al. showed that green (570 nm) and red (660 nm) LED irradiation increased *in vitro* fibroblast proliferation [32]. Low-dose irradiation at a specific wavelength is thought to stimulate and activate host cell function. For example, LED irradiation at different wavelengths in the range of 390–600 nm with low-radiant exposure of 0.04–50 J/cm^2 has been reported to stimulate healing and restore skin function [33].

The oxidative stress caused by ROS damages bacterial cell membranes, proteins, and genes [27,34–36]. Gene expression was analyzed to compare the effects of blue (450 nm) and red (625 nm) LED on *P. gingivalis*. We previously reported that 470-nm irradiation significantly decreased the expression of genes related to DNA replication and cell division [12]. In the present study, the expression of a gene related to DNA replication (*polA*), as well as genes related to chaperones (*htpG* and *clpB)* and the SOS response (*recA*), was reduced after 450 nm irradiation. Molecular chaperones promote protein folding and prevent protein misfolding and aggregation [37]. The SOS response in bacteria is a global response to DNA damage, in which the cell cycle is arrested, and DNA repair and mutagenesis are induced [38]. Our results suggest that 450-nm LED irradiation may decrease the broad general stress response during the stationary phase of *P. gingivalis*.

Gingipains are the major proteolytic enzymes of *P. gingivalis* and act as virulence factors in the development of periodontitis. Iron is essential for the growth of *P. gingivalis*, and gingipains are also closely linked to heme/iron acquisition from erythrocytes, promoting bacterial survival and proliferation [39,40]. In the present study, genes related to

gingipains (*rgpA, rgpB, kgp*) and hemin binding protein 35 (*hbp35*), which is related to hemin acquisition, were downregulated. These results indicate the inhibition of *P. gingivalis* growth and pathogenicity by blue LED irradiation. Yuan et al. recently reported that irradiation with 405-nm light at 30 J/cm^2 (100 mW/cm^2, 5 min) led to significant upregulation of *rgpA* and *rgpB*, as well as that of *ftn* and *fetB* [41]. This discrepancy may be attributed to the differences in the bacterial and light irradiation conditions used in the studies. It is also possible that even slight differences in light wavelength, bandwidth, and energy strongly affect bacterial activity.

In the present study, the expression of genes related to FimA fimbriae formation (*fimA, fimC*, and *fimD*) was also reduced after the irradiation of 450-nm LED light. To the best of our knowledge, this is the first study to examine the effects of blue LED irradiation without photosensitizers on FimA fimbriae genes. FimA is one of the main fimbriae of *P. gingivalis* and mediates bacterial colonization and biofilm formation in periodontal tissues. It also induces periodontal tissue inflammation through various pathways [42,43]. As mentioned above, the present study showed the reduction of gene expressions related to gingipains and hemin binding protein 35 (HBP35), which are involved in the formation and maturation of fimbriae [44]. Collectively, our results suggested that blue LED irradiation inhibits FimA fimbriae formation and attenuates bacterial activity.

LED irradiation at 625 nm increased the expression of genes related to DNA replication (*polA*) and homeostasis by degrading and removing abnormal membrane proteins (*ftsH*). These results suggest that the bacteria may recover from LED-induced damage and that red LED irradiation promotes these responses. While the expression of FimA-related genes tended to decrease, the present findings suggest that red light possibly alters bacterial pathogenicity without a bactericidal effect.

The present study showed that the effects of LED light on *P. gingivalis* and HGF-1 varied depending on the wavelength. Blue (450 and 470 nm) light showed antibacterial effects without a significant influence on the viability of HGF-1, whereas yellow-green (565 nm) and red (625 nm) light induced HGF-1 proliferation, which may promote periodontal tissue healing. These results show that the application of light with a combination of different wavelengths (*i.e.*, a combination of 450- and 625-nm LEDs) might be a novel method to achieve antibacterial effects and simultaneously promote periodontal tissue healing in clinical practice. A limitation of this study is that although altered expression of genes related to the pathogenesis of *P. gingivalis* was observed following blue LED irradiation, we have not yet verified the changes in the characteristics of this bacterium. In addition, we evaluated the effect of LED light only on *P. gingivalis* in this study. Indeed, *P. gingivalis* is a representative periodontopathic bacterium and a keystone pathogen that causes dysbiosis of the periodontal microbiome [45]; however, the oral cavity represents one of the largest microbiome in the human body, with more than 700 bacterial species [46]. The antibacterial effects and phototoxicity of LEDs against periodontal bacteria would vary depending on the bacteria because of different levels of endogenous photosensitizers. We plan to clarify these effects on multiple microorganisms in the future.

5. Conclusion

The effects of LED light on *P. gingivalis* and HGF-1 varied with the wavelength under the same irradiation conditions. When irradiated at 27 J/cm^2 (150 mW/cm^2, 3 min), UVA light (365 nm) showed the highest antibacterial effect and the highest cytotoxicity on HGFs. In contrast, blue light (450 and 470 nm) showed high antibacterial activity with low cytotoxicity, and using these wavelengths under the present irradiation conditions is promising for the elimination of *P. gingivalis* in periodontal therapy. In addition, blue light may attenuate the pathogenesis of *P. gingivalis*. Although yellow-green (565 nm) and red (625 nm) LEDs exerted no antibacterial effect, they may promote wound healing.

CRediT authorship contribution statement

Sakura Hayashi: Writing – original draft, Investigation, Funding acquisition, Formal analysis. **Yasuo Takeuchi:** Writing – review & editing, Supervision, Conceptualization. **Koichi Hiratsuka:** Writing – review & editing, Visualization. **Yutaro Kitanaka:** Investigation. **Keita Toyoshima:** Investigation. **Takashi Nemoto:** Investigation. **Nay Aung:** Investigation. **Masahiro Hakariya:** Writing – original draft, Investigation. **Yuichi Ikeda:** Writing – review & editing. **Takanori Iwata:** Writing – review & editing. **Akira Aoki:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

This work was supported by JST SPRING [grant number JPMJSP2120] and the Japan Society for the Promotion of Science KAKENHI [grant number 20K09971 to A. A.].

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Review

Application of Different Wavelengths of LED Lights in Antimicrobial Photodynamic Therapy for the Treatment of Periodontal Disease

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Abstract: Therapeutic light has been increasingly used in clinical dentistry for surgical ablation, disinfection, bio-stimulation, reduction in inflammation, and promotion of wound healing. Photodynamic therapy (PDT), a type of phototherapy, has been used to selectively destroy tumor cells. Antimicrobial PDT (a-PDT) is used to inactivate causative bacteria in infectious oral diseases, such as periodontitis. Several studies have reported that this minimally invasive technique has favorable therapeutic outcomes with a low probability of adverse effects. PDT is based on the photochemical reaction between light, a photosensitizer, and oxygen, which affects its efficacy. Low-power lasers have been predominantly used in phototherapy for periodontal treatments, while light-emitting diodes (LEDs) have received considerable attention as a novel light source in recent years. LEDs can emit broad wavelengths of light, from infrared to ultraviolet, and the lower directivity of LED light appears to be suitable for plaque control over large and complex surfaces. In addition, LED devices are small, lightweight, and less expensive than lasers. Although limited evidence exists on LED-based a-PDT for periodontitis, a-PDT using red or blue LED light could be effective in attenuating bacteria associated with periodontal diseases. LEDs have the potential to provide a new direction for light therapy in periodontics.

Keywords: phototherapy; antimicrobial photodynamic therapy; periodontal disease; light-emitting diode

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Citation: Takeuchi, Y.; Aoki, A.; Hiratsuka, K.; Chui, C.; Ichinose, A.; Aung, N.; Kitanaka, Y.; Hayashi, S.; Toyoshima, K.; Iwata, T.; et al. Application of Different Wavelengths of LED Lights in Antimicrobial Photodynamic Therapy for the Treatment of Periodontal Disease. *Antibiotics* **2023**, *12*, 1676. https://doi.org/10.3390/antibiotics12121676

Academic Editors: Andreas Braun and Felix Krause

Received: 17 October 2023
Revised: 23 November 2023
Accepted: 24 November 2023
Published: 28 November 2023

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1. Introduction

Optical technology has been used in medicine for examining, diagnosing, and treating patients. Particularly in current dental treatments, it has various applications, such as surgical ablation, disinfection, bio-stimulation, suppression of inflammatory responses, and promotion of wound healing (Figure 1) [1]. Compared with conventional mechanical therapies, phototherapy, a minimally invasive treatment method, has the advantage of reducing pain, discomfort, and tissue damage. It also accelerates wound healing, thereby improving the quality of life. Antimicrobial photodynamic therapy (a-PDT) is a modern form of phototherapy, which has garnered attention as a new therapeutic method for

eradicating oral bacterial infections, and its application in periodontal treatment has been investigated [2,3]. a-PDT has been predominantly performed using lasers for the treatment of periodontal infections, and its adjunctive effect when combined with non-surgical periodontal debridement has also been evaluated. Light-emitting diodes (LEDs) have been used as a new light source for a-PDT, and the number of studies on a-PDT using LEDs has increased in recent years. This review presents an overview of the light application in periodontal treatments and describes the potential application of different wavelengths of LED as a new light source in a-PDT/phototherapy for the management of periodontal diseases.

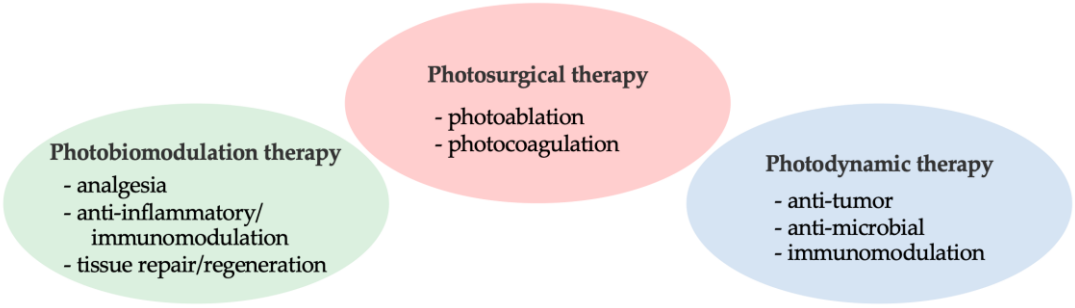

Figure 1. Various therapeutic effects obtained with the application of light.

2. Periodontal Diseases

Periodontal disease is an inflammatory condition affecting the periodontium, which leads to the destruction of periodontal tissues and eventual tooth loss. Approximately 1.1 billion individuals worldwide are reportedly affected by severe periodontal disease [4]. Recent epidemiological, clinical, and animal model studies have suggested a possible link between periodontal disease and various systemic diseases, such as diabetes and cardiovascular disease. Preventing and controlling periodontal disease is essential for maintaining oral and systemic health [5–7]. Although various environmental factors and systemic conditions may be involved in the onset and progression of diseases, bacterial plaque is generally considered the primary etiological factor. Mechanical instrumentation is considered in initial periodontal therapy for eliminating plaque and calculus deposits. However, conventional mechanical therapy may not entirely eliminate these etiologic factors in areas with limited access, such as deep pockets and complicated root surfaces. Antibiotic and antiseptic chemotherapies have limited efficacy against dental plaque residing as a biofilm [8,9], and the emergence of antibiotic-resistant bacteria is concerning [10,11].

3. Phototherapy in Periodontics

Optical technology is widely used for treating oral diseases, especially in the field of periodontics. Lasers, a monochromatic collimated beam of light, which can be concentrated into powerful energy beams within a narrow range, have been used as light sources for phototherapy in periodontics. High-power laser devices have been used to selectively cut hard and soft tissues and further remove diseased tissues. In particular, erbium-doped yttrium aluminum garnet (Er:YAG) laser is used to remove calculus in periodontal therapy [12,13]; the laser irradiation enables minimally invasive debridement and neutralization of diseased root surfaces. Photobiomodulation (PBM) uses low-power laser irradiation to trigger photophysical and photochemical reactions in host cells, resulting in beneficial physiological changes. Several studies have demonstrated the ability of PBM to promote rapid wound healing and alleviate pain at diseased sites [14–16]. Photodynamic therapy (PDT), which uses light and light-activatable photosensitizers, has garnered significant interest in recent years, with an increase in the number of basic and clinical studies on PDT.

PDT was originally developed as a treatment technique for cancers in various organs and age-related maculopathy [17,18]. PDT uses a specific wavelength of light to irradiate an area after administering a photosensitive substance, which has an affinity for the target tumor cells. The application of light excites the substance, and this process triggers the

production of reactive oxygen species (ROS), leading to the destruction of the lesioned tissue. On the other hand, PDT used specifically to eradicate bacteria is known as "antimicrobial photodynamic therapy (a-PDT)". a-PDT has been used for the neutralization of harmful substances resulting from bacterial adherence to the root surfaces [19,20]. In vitro treatment with a-PDT potently and functionally inactivates inflammatory mediators, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which can impair periodontal restoration; thus, photochemical reactions from a-PDT may have the potential to enhance periodontal treatment [20]. The use of a-PDT has raised concerns regarding adverse effects on host cells. However, the light intensity used for bacterial elimination or suppression is below the toxicity limit of the host cell. Moreover, the light used during a-PDT exerts photobiological effects on the surrounding tissues, thereby providing important benefits for the periodontal healing process [1]. Although the mechanism is still debated, irradiated light reduces inflammation by regulating immunocompetent cells and supports the healing of periodontal tissues [21–29].

a-PDT is useful for sites, which are difficult to access with mechanical instruments. Several clinical studies have examined the effectiveness of a-PDT as an adjunctive therapy after conventional mechanical debridement of periodontal disease. Diode lasers have been used as light sources for a-PDT in the majority of studies. However, the discrepancies in the application conditions of a-PDT across studies have led to some researchers reporting inconsistent results, which limit the proven effectiveness of the adjunct use of a-PDT in the treatment of periodontal disease [30–32]. A 2021 systematic review by Moro et al. [33] evaluated 22 studies, which used a-PDT as an adjunctive therapy to scaling and root planing (SRP) with a 3-month follow-up. The results showed that the combination of a-PDT and SRP led to a significant increase in the clinical attachment level (CAL) and a decrease in the periodontal probing depth (PD) compared with those of SRP alone. They also found that the clinical efficacy of a-PDT was high when indocyanine green or high-concentration phenothiazine chloride was used as the photosensitizer. The beneficial effects of a-PDT also include reducing periodontopathic bacteria and improving clinical parameters [2].

Laser devices have been used as light sources in phototherapy for periodontal treatments. However, the activation of the photosensitizers used in a-PDT does not necessarily require high power light of lasers, and studies employing LEDs as light sources have increased in recent years. Similar to lasers, LEDs can be applied using a fiber-type tip to effectively irradiate anatomically complex areas, such as deep periodontal pockets. The lower directivity of LED light compared with that of lasers enables the irradiation of large and complicated surfaces using a flashlight-type device. Furthermore, LED devices are small, lightweight, and less expensive than lasers, making the production of home-use LED products for daily plaque control easier.

4. Antimicrobial Effects of Various Wavelengths of LEDs

LED devices can output wavelengths of light in the ultraviolet (UV), visible, and infrared regions. Several major periodontopathic bacteria possess endogenous photosensitizers, which are inactivated by the wavelength of LED light irradiation alone [34–36]. However, more than 700 bacterial species reside in the oral cavity, and the combined use of LED lights and photosensitizers is necessary to exert an antibacterial effect against various micro-organisms in clinical practice. The antibacterial effect of a-PDT is influenced by several factors, such as the dose and type of photosensitizer, wavelength and irradiance of light, and oxygen content in the irradiation field. A higher antibacterial effect can be expected when the wavelength of the irradiation light matches the absorption wavelength of the photosensitizer. Most photosensitizers can adhere to both bacteria and periodontal tissues and exhibit toxicity to the bacteria even in the absence of light [37]. The clinical application of a-PDT in periodontal treatment requires high antibacterial effects to be accompanied by minimal damage to host cells. The number of clinical studies investigating antibacterial phototherapy using LEDs is substantially lower than that of studies using

lasers; however, in vitro studies examining the antibacterial effects of various wavelengths of LED lights with and without the use of photosensitizers have been reported increasingly.

4.1. Search Strategy

An electronic search of PubMed/MEDLINE, Cochrane Central Register of Controlled Trials (CENTRAL), and Web of Science databases was conducted for studies in March 2023. The search was limited to literature published in English. Literature searches in these databases were performed using the following terms: ("periodontal diseases" OR "periodontitis" OR "gingivitis" OR "periodontics" OR "periodontal") AND ("light-emitting diode" OR "light emitting diode" OR "LED") AND ("phototherapy" OR "photodynamic" OR "photodynamic therapy" OR "antimicrobial photodynamic therapy" OR "antibacterial photodynamic therapy" OR "a-PDT" OR "PDT"). An additional manual search of the references listed in all the included articles was conducted to identify further possible articles. The literature resulting from the search was screened by the authors first on the basis of titles and abstracts, and subsequently, on the basis of full texts. Particularly in clinical studies, the full-length articles of studies were evaluated in detail for inclusion in this review, and case reports/series, studies investigating the effect of LED light irradiation alone without photosensitizer, and studies targeting the treatment of peri-implant disease were excluded. Data extracted from the clinical research papers using LED-based a-PDT listed in Table 1 included publication information, study design, sample size, photosensitizer type/concentration, irradiation parameters and regimen, investigated parameters, and follow-up period.

4.2. Red Light/Infrared Light

The use of red light as an excitation light in a-PDT for periodontitis has been studied extensively. Phenothiazine-based photosensitizers, such as toluidine blue and methylene blue, show absorption peaks around 600–700 nm and are frequently used in combination with red light in a-PDT for periodontal diseases. The increased proportion of Gram-negative anaerobic bacteria in the dysbiotic microbiota of the diseased sites in the periodontium and the relatively small molecular weight and structural cationic charge of these photosensitizers facilitate easy penetration into the outer membrane of both Gram-negative and Gram-positive bacteria with a high affinity.

Red/infrared diode laser was used as the light source for a-PDT with blue dye initially, and its antibacterial effect against Gram-negative periodontopathic bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, has been demonstrated in vitro. Additional studies using red/infrared LED light have been conducted to confirm their efficacy [38,39]. Commercially available laser devices specifically designed for a-PDT in dental treatment were released before LEDs, and most clinical studies have used diode lasers. The clinical efficacy of a-PDT using red or near-infrared laser light with blue dye as an adjunctive therapy to mechanical debridement has been evaluated during the non-surgical and supportive phases of periodontal therapy. Wavelengths of 660 or 810 nm (radiant exposure: 0.6 to 1414.7 J/cm^2) were used with photosensitizers in these studies, and additional beneficial effects on clinical parameters and a reduction in the bacteriological burden were observed [2]. It has also been shown to be effective in combination with mechanical debridement during periodontal surgery [40].

Table 1. Clinical studies of LED-based antimicrobial photodynamic therapy in the treatment of periodontitis.

Reference	Study Participants (n) Study Design	Groups (n: Sites) Treatment Provided	Follow-Up Findings
Bassir et al., 2013 [41]	CP (16) Split-mouth	• SRP + a-PDT (119): SRP (US/H) +LED (625–635 nm, 2000 mW/cm^2, Fotosan®) +TB (0.1 mg/mL) Photosensitizer incubation time: not described Irradiation time: 10 s (inside) + 10 s (outside the pocket), ×6 (per tooth) • SRP + LED (96): SRP (US/H) +LED (625–635 nm, 2000 mW/cm^2, Fotosan®) Irradiation time: 10 s (inside) + 10 s (outside the pocket), ×6 (per tooth) • SRP + PS (90): SRP (US/H) + TB (0.1 mg/mL) • SRP (91): SRP (US/H)	• Follow-up: 3 months. • Adjunctive treatments (a-PDT, LED, or PS) were repeated 7 and 14 days later. • Photoactivation using LED did not show additional effects on clinical parameters (PD, CAL, BOP, PI) compared to SRP alone.
Pulikkotil et al., 2016 [42]	CP (16) Split-mouth	• SRP + a-PDT (16): SRP + LED (628 nm, Fotosan®) + MB Photosensitizer incubation time: 1 min Irradiation time: 10 s (inside) + 10 s (outside the pocket) • SRP (16): SRP	• Follow-up: 3 months. • Significantly greater improvement in BOP was seen in the SRP + a-PDT group compared to that in the SRP group after 3 months of treatment. • No difference in the quantity of Aa was detected between the groups.
Husejinagic et al., 2019 [43]	Periodontitis (20) Split-mouth, RCT	• SRP + a-PDT (20): SRP (US/H) +LED (635 nm, 750 mW, PADPLUS) + TB (12.7 µg/mL) Photosensitizer incubation time: 1 min Irradiation time: 10 s (inside the pocket), ×6 (per tooth) • SRP (20): SRP (US/H)	• Follow-up: 3 months. • Significant improvements in clinical parameters (PD, CAL, BOP) were shown in both groups, but the test and control groups were comparable. • The recolonization of Pg and Td was reduced after adjuvant treatment, but not significantly.

Table 1. *Cont.*

Reference	Study Participants (n) Study Design	Groups (n: Sites) Treatment Provided	Follow-Up Findings
Harmouche et al., 2019 [44]	Periodontitis (28) Split-mouth, RCT	• SRP + a-PDT (579): SRP (US/H) +LED (625–635 nm, 2000 mW/cm^2, Fotosan®) +TB (0.1 mg/mL) Photosensitizer incubation time: 1 min Irradiation time: 10–30 s (inside) + 10 s (outside the pocket) PDT applications were repeated 7 days and 3 months after SRP. • SRP (609): SRP (US/H)	• Follow-up: 6 months. • Repeated application of a-PDT with SRP significantly improved SRP outcome (PD and BOP) compared to SRP alone. • This effect was mainly observed at 6 months in initially deep sites (PD > 6 mm) with BOP.
Mongardini et al., 2014 [45]	CP (30); Residual pockets during SPT Split-mouth	• SRP + a-PDT (30): SRP (H) +LED (628 nm, 2000 mW/cm^2, Fotosan®) + TB (0.1 mg/mL) Photosensitizer incubation time: 1 min Irradiation time: 10 s (outside) + 10 s (inside the pocket), ×2 (per tooth) • SRP (30): SRP (H)	• Follow-up: 1 week. • One week after the treatment, the number of sites showing a PD reduction of ≥2 mm was higher in the a-PDT group than in the SRP group. • Higher reductions in relative proportions of red complex bacteria were observed in the a-PDT group compared to the SRP group.
Goh et al., 2017 [46]	Periodontitis (27); Residual pockets during SPT Split-mouth, RCT	• SRP + a-PDT (36): SRP (US/H) +LED (620–640 nm, 2000–4000 mW/cm^2, Fotosan®) +TB (0.1 mg/mL) Photosensitizer incubation time: not described Irradiation time: 20 s/site × 2 • SRP (36): SRP (US/H)	• Follow-up: 6 months. • At 3 months after treatment, significantly greater improvements in PD and CAL were observed in the SRP + a-PDT group compared to the SRP group. However, the differences were no longer significant at the 6-month follow-up. • Adjunctive a-PDT did not offer additional reduction in the levels of GCF cytokines, including IL-8, IL-6, and TNF-α.

Table 1. *Cont.*

Reference	Study Participants (n) Study Design	Groups (n: Sites) Treatment Provided	Follow-Up Findings
Hormdee et al., 2020 [47]	Periodontitis (12) Split-mouth, RCT	• SRP + a-PDT (12): SRP (US/H) +LED (420–480 nm, 1000–1200 mW/cm^2) +CUR gel (25 µg/mg) Photosensitizer incubation time: none (irradiated immediately) Irradiation time: 2 min (inside the pocket) per tooth • SRP (12): SRP (US/H)	• Follow-up: 6 weeks. • In the a-PDT group, significant reductions in PD and CAL were observed in the intragroup comparison from the first week up to the fourth week of follow-up. In contrast, a significant reduction in PD was observed after only a week in the SRP group. • The quantities of Fn and Pi were recovered in the SRP group, while there was no significant recolonization of these bacteria on PDT sites throughout the 6-week study duration.
Ivanaga et al., 2019 [48]	CP with type 2 DM (23); Residual pockets during SPT Split-mouth, RCT	• SRP + a-PDT (88): SRP (US/H) +LED (465–485 nm, 100 mW/cm^2, InGaN) + CUR (0.1 mg/mL) Irrigation with CUR for 1 min Irradiation time: 60 s (outside the pocket) • SRP + LED (80) SRP (US/H) +LED (465–485 nm, 100 mW/cm^2, InGaN), Irrigation with 1 mL saline solution Irradiation time: 60 s (outside the pocket) • SRP + PS (67): SRP (US/H) + CUR (0.1 mg/mL) • SRP (97): SRP (US/H) Treatment modalities were performed in a total of 332 sites, but only 290 sites were included in the final evaluation.	• Follow-up: 6 months. • Significant improvements in PD and BOP were shown in all treatment groups; however, the mean values for PD, CAL, GR, BOP, and PI did not differ among the four groups at baseline, 3-, and 6-month follow-ups. • Significant CAL gain was found only in the a-PDT and LED groups at 3 months in comparison to baseline data.

CP: chronic periodontitis, SRP: scaling root planing, H: hand scaling, US: ultrasonic scaling, a-PDT: antimicrobial photodynamic therapy, PS: photosensitizer, LED: light-emitting diode, MB: methylene blue, TB: toluidine blue, CUR: curcumin, PD: probing depth, CAL: clinical attachment level, GR: gingival recession, BOP: bleeding on probing, PI: plaque index, GI: gingival index, Aa: *Aggregatibacter actinomycetemcomitans*, Fn: *Fusobacterium nucleatum*, Pg: *Porphyromonas gingivalis*, Pi: *Prevotella intermedia*, Td: *Treponema denticola*, GCF: gingival crevicular fluid.

Conversely, a limited number of clinical studies have been conducted on a-PDT utilizing red LED light (Table 1). Most of these studies employed commercially available red LED devices (peak wavelength of 620–640 nm, 2000 mW/cm^2) with photosensitizers and similar irradiation conditions [20 J/cm^2/site × 2 from inside and outside the pocket, applying toluidine blue (0.1 mg/mL) prior to irradiation] and have demonstrated the efficacy of a-PDT as an adjunct to conventional mechanical treatment. However, several studies have reported conflicting results. For instance, Bassir et al. [41] examined the efficacy of LED-based a-PDT combined with conventional debridement in 16 patients with moderate-to-severe periodontitis and reported no additional benefits of a-PDT for the clinical outcomes compared with SRP alone. The discrepancies in the results may be due to differences in patient characteristics and a-PDT procedures (e.g., application time of photosensitizers and irradiation technique).

The morphology of the roots to which the bacteria are attached is complex. Periodontal pockets become deeper as periodontal disease progresses, making it increasingly difficult to align the light port with the target area even when a fiber-type tip is used. Red light is on the long-wavelength side of visible light and penetrates the gingiva to a greater extent. The penetration depth of light at the wavelength of 660 nm, which is commonly used in a-PDT, is approximately 3.0–3.5 mm [49]. Although light energy is attenuated as it passes through the gingiva [50,51], red light can act on the bacteria in the pockets, even when irradiated from the gingival surface. Transgingival irradiation facilitates easier and faster treatment without the insertion of the light into the periodontal pocket. In a study using a diode laser, Sasaki et al. [52] used a gingival model created using beef slices to determine the feasibility of transgingival a-PDT. They reported that a combination of indocyanine green-encapsulated nanoparticles (final concentration: 10 mg/mL) and infrared light from a diode laser (810 nm, 960–4800 J/cm^2) had an antimicrobial effect even with transgingival irradiation. Schär et al. [53] evaluated the clinical effects of transgingival irradiation with red diode laser light [670 nm, 39 or 58 J/cm^2 (single root tooth or molar)] and 1% methylene blue after SRP in periodontal pockets of patients with stage II–III periodontitis. They found that compared with the control group, the test group showed a significant reduction in bleeding on probing (BOP) and a trend toward improvement in PD and CAL after treatment. Compared with lasers, LED light is more suitable for irradiating a relatively wide area of the gingiva. The authors [54] also showed that a-PDT with red LED light (660 nm, 1.1 W/cm^2, 20 s/site) and toluidine blue (1 mg/mL) inhibited plaque formation. Wide-area irradiation using LEDs is easy to implement and can be applied for plaque control (Figure 2).

4.3. Blue Light

Short-wavelength lights, such as blue light, lack the ability to penetrate deeply into tissues; thus, they cannot be used as light sources for PDT, which targets tissues deep in the human body. However, fiber-shaped tips have enabled the direct irradiation of periodontal pockets, making it possible to apply short-wavelength light for a-PDT in periodontal treatment and supportive care. Blue light can be generated by diodes or argon lasers and has been used in clinical dentistry for bleaching and curing resins. However, the equipment cost limits its widespread use. In contrast, blue LED devices are less expensive than laser devices and are now widely used to cure resin materials used for restoring dental caries. Research on the benefits of blue LED light with the aim of applying it to phototherapy is ongoing.

Curcumin (maximum absorption wavelength: 425 nm), riboflavin (266 nm, 373 nm, 445 nm), rose bengal (550 nm), erythrosine (530 nm), and sinoporphyrin sodium (366 nm) have been used as photosensitizers in a-PDT using third-generation blue LED light [55]. The antibacterial effect of a-PDT in combination with blue LEDs and the aforementioned photosensitizers has been demonstrated in vitro on periodontal bacteria commonly found in patients with periodontitis, such as *A. actinomycetemcomitans*, *Campylobacter rectus*, *Fusobacterium nucleatum*, *Parvimonas micra*, *P. gingivalis*, *Tannerella forsythia*, *Prevotella intermedia*,

and *Streptococcus gordonii* [56–65]. In addition, the antibacterial activity of blue light irradiation alone has been demonstrated against some oral bacteria (Figure 3) [36,66–71], further MRSA, and novel coronavirus [72,73]. Black-pigmented bacteria, including *P. gingivalis* and *P. intermedia*, which are commonly associated with periodontal diseases, degrade hemoglobin to derive heme, and endogenous porphyrin is produced when the bacteria further acquire iron from heme [74]. The importance of porphyrins in the antimicrobial action of blue light has been suggested [74]; irradiated blue light reacts with endogenous porphyrin as a photosensitizer, leading to the production of ROSs, which efficiently and selectively kill bacteria [3,34,57,68,75]. This characteristic could have clinical advantages, as blue light exposure without a photosensitizer suppresses only the growth of periodontal pathogenic bacteria possessing endogenous porphyrin while simultaneously minimizing the impact on the natural microbiome. Masson-Meyers et al. [76] compared the antibacterial effects of 405 nm LED and 405 nm diode laser irradiation with the same amount of energy against *Staphylococcus aureus* and reported that both light therapies significantly suppressed bacterial growth and that the differences between the two light sources did not affect the results. Although the phases of LED light are not aligned with those of laser light, the total energy may have a greater influence on the outcome in terms of antibacterial efficacy. Blue light is more readily absorbed by the human mucosal tissue than red light [49], and excessive exposure to high-power blue light can cause tissue damage. Therefore, it is necessary to carefully consider the irradiation conditions for clinical use, although direct irradiation of the pocket can minimize the effect on host cells, which is advantageous from a safety standpoint.

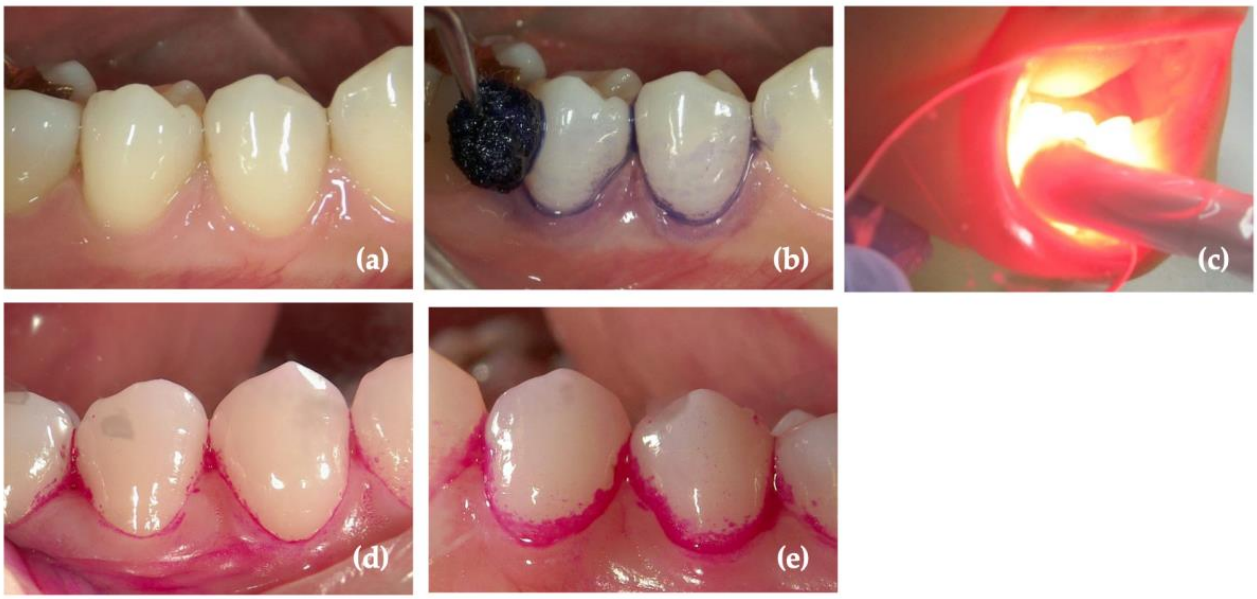

Figure 2. Suppression of dental plaque formation after antimicrobial photodynamic therapy (a-PDT) (red light-emitting diode (LED)/toluidine blue). Dental plaque was removed from tooth surfaces (**a**); toluidine blue O (1 mg/mL) was gently applied (**b**). After washing, red LED (660 nm, 1.1 W/cm^2) was focused on the tooth surfaces (**c**). Suppression of plaque formation on the a-PDT group teeth (**d**) could be confirmed compared to the control teeth (**e**). Figure from Ichinose-Tsuno, A et al. Antimicrobial photodynamic therapy suppresses dental plaque formation in healthy adults: a randomized controlled clinical trial. *BMC Oral Health* **2014**, 14, 152. CC-BY 4.0 [14].

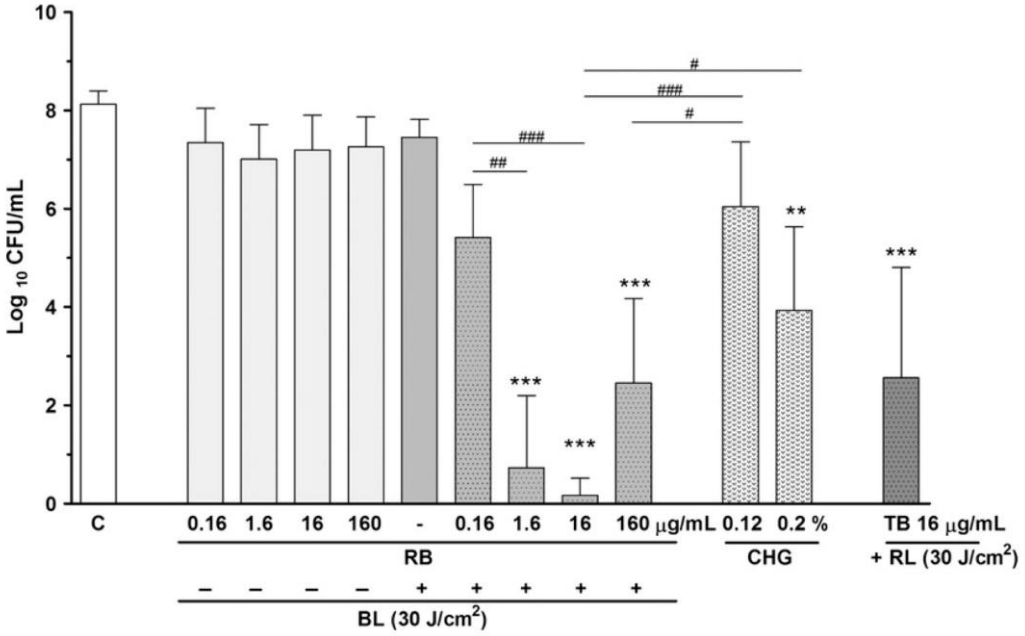

Figure 3. Effect of blue light-emitting diode (LED) (BL) in combination with graded rose bengal (RB). Fresh *Porphyromonas gingivalis* suspension was mixed with RB (the final concentrations were 0.16, 1.6, 16, and 160 μg/mL, respectively) and irradiated with 30 J/cm^2 BL. Bacterial suspensions treated with chlorhexidine gluconate (CHG) or other a-PDT [a combination of toluidine blue (TB) and red LED (RL) irradiation] were employed as the positive controls. After the treatment, each suspension was plated and incubated on brucella agar plates, and the numbers of colony-forming units (CFU) were determined. a-PDT using BL and RB showed a high antibacterial effect against periodontopathic bacteria in vitro. ** $p < 0.001$, *** $p < 0.0001$ (vs. Control). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.001$, $^{\#\#\#}$ $p < 0.0001$. Figure from Chui, C et al. Antimicrobial effect of photodynamic therapy using high-power blue light-emitting diode and red-dye agent on *Porphyromonas gingivalis*. *J Periodontal Res* **2013**, 48, (6), 696–705. © Copyright (2013) John Wiley & Sons A/S. DOI: 10.1111/jre.12055 [36].

Only a few clinical studies have evaluated the effects of a-PDT using blue LED (Table 1). Hormdee et al. [47] investigated the efficacy of a-PDT using blue LED and *Curcuma longa* extract as adjuncts to SRP in the treatment of patients with moderate chronic periodontitis. The application of *Curcuma longa* gel (concentration: 25 μg/mg) after SRP followed by irradiation of the pockets with blue LED (16.8 J/cm^2) led to a significant improvement in PD and BOP at 4 weeks after treatment. The number of periodontopathic bacteria (*F. nucleatum* and *P. intermedia*) was also significantly suppressed after treatment. The probing depth decreased significantly one week after treatment in the control group; however, recurrence of the periodontal pocket with recolonization of periodontopathic bacteria was observed thereafter. Ivanaga et al. [48] investigated the clinical efficacy of a-PDT with curcumin solution (concentration: 0.1 g/L) and blue LED light (465–485 nm, 7.69 J/cm^2, irradiated outside of the pockets) as adjunctive therapy to SRP in residual pockets after periodontal therapy in patients with periodontal disease and type 2 diabetes. A reduction in PD and BOP positivity rates was observed in the SRP-only and a-PDT combined groups after treatment. However, significant CAL gain was observed only in the a-PDT combined group at three months in comparison to baseline data. Araújo et al. [77] used a mouthwash containing a curcumin solution (concentration: 1.5 g/L) and irradiated the mouths of 13 volunteers with blue LED (450 nm, 20.1 J/cm^2, irradiated the oral cavity by inserted light source) to determine the effectiveness of a-PDT for routine oral hygiene purposes. They examined the quantity of bacteria in the saliva before and after treatment and reported that a-PDT using curcumin mouthwash and blue LED significantly reduced the bacteria (68.3%), whereas the use of mouthwash alone did not result in an effective reduction in bacteria (9%). Ricci Donato et al. [78] instructed 50 volunteers to rinse with one of two

photosensitizers (curcumin or a hematoporphyrin derivative). Light illumination (blue light at 450 nm for curcumin and red light at 630 nm for the hematoporphyrin derivative; irradiance: <100 W/cm^2 for 6 min) was performed using a LED device. The results showed a significant reduction in the number of bacteria in the saliva after both photodynamic treatments; a reduction was observed even after 24 h of treatment in the curcumin + blue light group. Genina et al. [79] investigated the efficacy of a toothbrush equipped with a blue light-emitting function (405–420 nm, 2 mW/cm^2) as a daily means of suppressing the progression of periodontal disease. Sixty participants with mild-to-moderate gingivitis were randomly divided into two groups (blue LED-emitting toothbrushes and regular toothbrushes). A reduction in the accumulation of dental plaque and gingival inflammation was observed in both groups after using the toothbrushes for one month; however, the efficiency (improvement in clinical indices) of brushing was significantly higher in the LED toothbrush group than in the control group.

Only a limited number of clinical studies have been conducted on a-PDT using blue LEDs, and there is heterogeneity in study designs. Thus, it is difficult to determine its clinical efficacy at present; however, many ongoing studies have suggested a growing potential for the clinical application of blue light.

4.4. Green/Yellow Light

Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,77-tetraiodofluorescein, RB) is present as a pigment in plaque-disclosing agents used in dentistry. a-PDT combined with RB as the photosensitizer and blue LED light (425–470 nm) exhibits high antibacterial activity against *P. gingivalis* in vitro (Figure 3) [58]. The maximum absorption wavelength of RB is approximately 550 nm. Therefore, Kitanaka et al. [80] investigated the antibacterial effects of a-PDT using a new combination of yellow-green LED light (565 nm) and RB against *P. gingivalis* in vitro. The results showed that a-PDT with yellow-green LED (8.56 J/cm^2) and RB showed higher antibacterial activity against *P. gingivalis* than a-PDT with blue LED light (470 nm, 8.56 J/cm^2) and RB. In addition, morphological changes suggesting leakage of bacterial contents were observed under a scanning electron microscope within a short period of time after a-PDT; no subsequent bacterial growth was observed (Figure 4). These results suggest that a-PDT with RB and yellow-green LED resulted in the physical destruction of the bacterial cell wall and high bactericidal activity. Green light has also been reported to have photobiomodulatory effects on osteoblasts and bone cells and has attracted considerable attention [81]. However, to the best of our knowledge, the effects of a-PDT using green or yellow-green wavelengths of light have not been clinically evaluated in dentistry, and this area of research is expected to progress in the future.

4.5. UV Light

The peak of light absorption for DNA is at 260 nm. UVC (200–280 nm) and UVB (280–320 nm) can induce the formation of pyrimidine-pyrimidone (6-4) photoproducts or cyclobutane-type pyrimidine dimers, which damage DNA [82–84]. The bactericidal effect of UV light on oral bacteria is thought to be mediated via a similar mechanism. In vitro studies have shown that UV-LED light has a bactericidal effect against oral bacteria, such as *F. nucleatum*, *P. gingivalis*, *S. mutans*, and *S. sanguinis* [85,86]. The antibacterial action of UV light alone (without the use of a photosensitizer) was evaluated in these studies (Figure 5). UVC-LED showed a strong bactericidal effect; however, high cytotoxicity was demonstrated in a cell viability test using gingival fibroblasts [86]. UVB-LED exhibited a weaker bactericidal effect on oral bacteria than UVC-LED; however, it showed lower cytotoxicity to gingival epithelial cells. UVB-LED may induce the production of ROS from oral epithelial cells and enhance bactericidal activity against specific periodontopathic bacteria, such as *P. gingivalis* [85]. These results suggest that UVB-LED can also be used to control infections in the oral cavity.

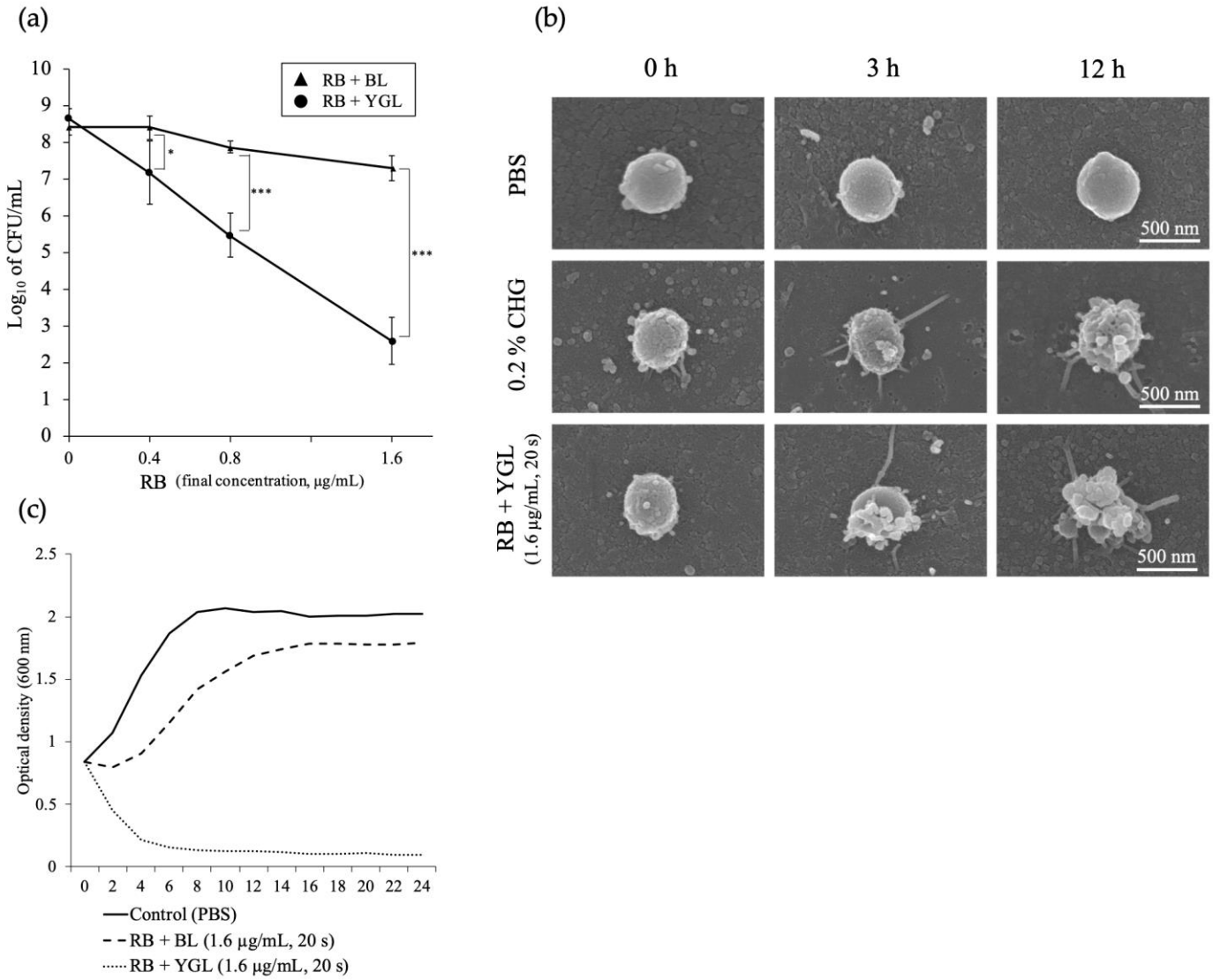

Figure 4. Antibacterial effects of a-PDT (rose bengal (RB) plus yellow-green light-emitting diode (LED) (YGL)) on *Porphyromonas gingivalis* (Pg). Pg suspension was mixed with RB and irradiated with YGL or BL for 20 s (8.56 J/cm^2) in vitro. (**a**) Antimicrobial photodynamic therapy (a-PDT) treatment employing RB + YGL significantly decreased viable bacteria as compared to RB + BL. (**b**) Morphological change in Pg was noticeable after a-PDT treatment (RB and YGL). (**c**) After the treatment with RB + BL, the Pg growth was inhibited temporarily and then increased to a plateau. In contrast, a dramatic reduction in Pg growth rate in the RB + YGL group was observed up to 6 h, finally reaching the lowest plateau. * $p < 0.05$, *** $p < 0.001$. Figure from Kitanaka, Y et al. The effect of antimicrobial photodynamic therapy using yellow-green LED and rose bengal on Porphyromonas gingivalis. *Photodiagnosis Photodyn Ther* **2020**, 32, 102033. © Copyright (2020) Elsevier. DOI: 10.1016/j.pdpdt.2020.102033 [80].

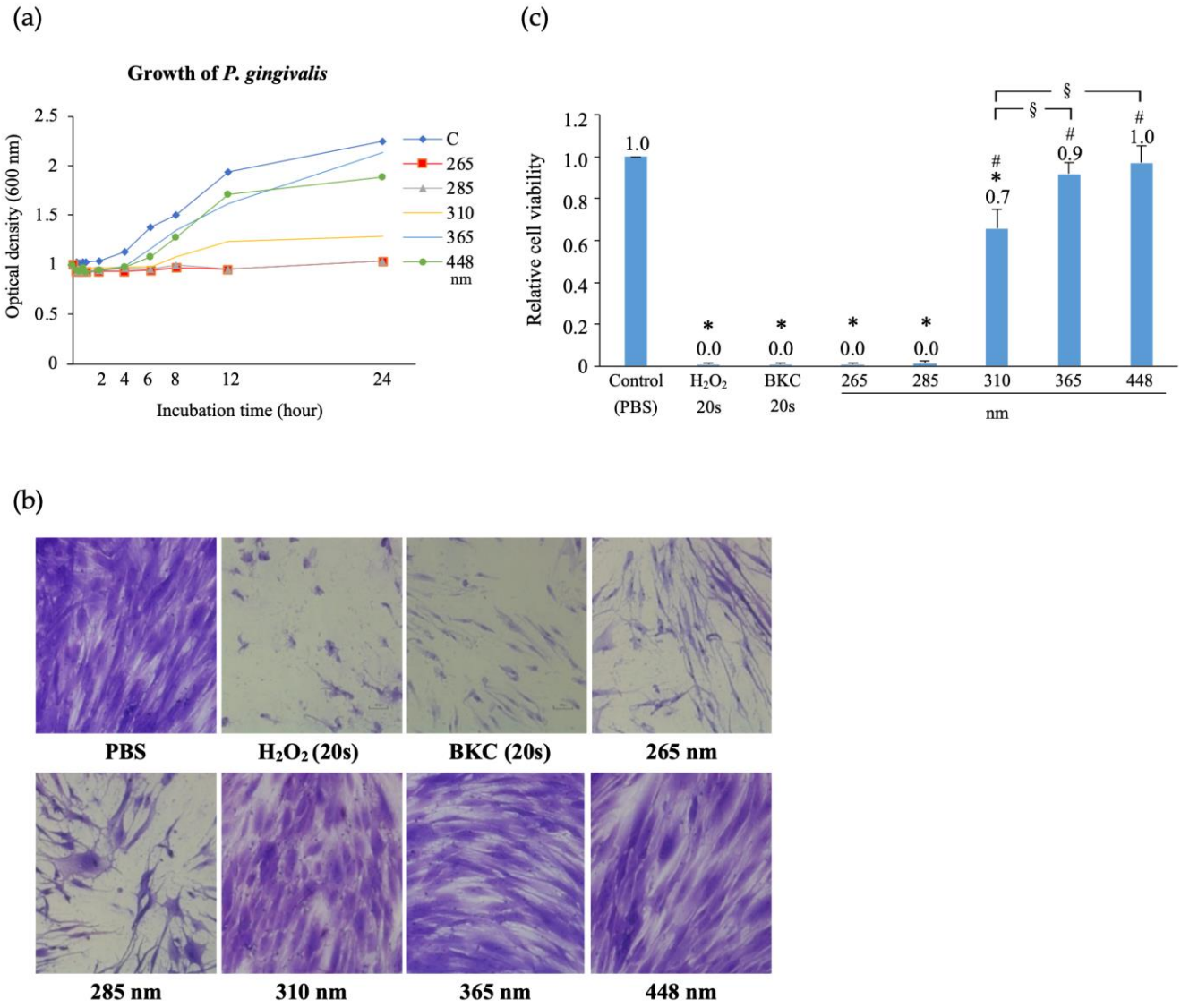

Figure 5. Effect of ultraviolet (UV) and blue light-emitting diode (LED) light on *Porphyromonas gingivalis* (Pg) and human gingival fibroblasts. (**a**) Growth of Pg after anaerobic irradiation (600 mJ/cm^2) with UV and blue LED light. Pg irradiated at 265 or 285 nm showed little growth over 24 h, while a slight growth increase was observed with 310 nm irradiation. (**b**) Crystal violet staining images of human gingival fibroblasts 24 h after UV-LED irradiation. (**c**) Viability of human gingival fibroblasts 24 h after UV-LED irradiations. Oral antiseptic agents [3% H_2O_2 (20 s) and 0.025% benzalkonium chloride (BKC) (20 s)] and phosphate-buffered saline (PBS) were used as controls. Irradiation with UVB light (265 or 285 nm, 60 s) completely devitalized HGF-1. Irradiation at 310 nm reduced viability by 30% after 24 h, whereas irradiations at 365 and 448 nm resulted in no significant reduction compared with control group. * $p < 0.05$ (vs. Control), $^{\#}$ $p < 0.05$ (vs. H_2O_2, BKC, 265, and 285 nm groups), § $p < 0.05$. Figure from Aung, N et al. The effects of ultraviolet light-emitting diodes with different wavelengths on periodontopathic bacteria in vitro. *Photobiomodul Photomed Laser Surg* **2019**, 37, (5), 288–297. © Copyright (2019) Mary Ann Liebert, Inc. DOI: 10.1089/photob.2018.4514 [86].

Narrow-band (NB)-UVB light, the specific wavelength around 310 nm with a narrow peak, has already been used to treat skin diseases—including psoriasis, vitiligo vulgaris, and atopic eczema—in dermatology. NB-UVB has fewer side effects on the host than broad-band-UVB light [87–89] and has been reported to upregulate regulatory T cells [90]. The immunosuppressive effects of NB-UVB may also be preferable for the treatment of periodontitis [91–94]. However, at present, few studies have investigated the application of

UVB-LED light in a-PDT or phototherapy for periodontal treatments, and the efficacy and safety should be carefully investigated through in vitro and clinical trials before applying it in clinical practice. UVB-induced immunosuppression may facilitate oral microbial infection. Over-irradiation with UVB light can cause damage to oral tissues and increase the risk of oral cancer [95,96].

5. Future Perspectives and Conclusions

Mechanical debridement at the site of infection remains the gold standard of treatment for periodontal diseases. However, phototherapy may be a useful adjunct to conventional therapy in anatomically complex areas and areas with limited access. a-PDT is a minimally invasive and virtually painless treatment, which does not produce antibiotic-resistant bacteria.

Although the irradiance of LEDs is generally low compared with that of lasers, a certain low threshold of light energy may be sufficient to induce bactericidal effects using a-PDT. LEDs generally have a low heat output; however, when used at high irradiance during treatment, the excessive heat generated by LEDs causes discomfort to patients. Moreover, there is a possibility of phototoxicity to neighboring tissues, which are part of the irradiation field. Therefore, further in vitro, in vivo, and clinical studies are required to determine the optimal irradiance, irradiation time, and other aspects to ensure the safety and effectiveness of LED phototherapy. To promote the clinical application of LED-based a-PDT, it is necessary to perform in vitro experiments under standardized irradiation conditions and establish recommended a-PDT protocols, which are presumed to be highly effective based on basic research data.

The wavelength of the irradiated light should be matched with the wavelength, which can be absorbed by the photosensitizer, particularly when performing a-PDT. LEDs produce light with various peak wavelengths within a narrow bandwidth. Thus, LEDs can provide light at an optimal wavelength for any photosensitizer (including endogenous photosensitizers in the pathogenic bacterial cells) used in phototherapy. However, simply increasing the dose of light or the concentration of a photosensitizer may not improve the effect of a-PDT [97,98]. Photochemical reactions and the characteristics of photosensitizers (i.e., the toxicity of the photosensitizer itself) also affect the results of a-PDT. A higher concentration of the dye can cause dimerization or multimerization of the photosensitizer and shift the absorption wavelength peak of the dye. The mismatch between the wavelengths of the light and the photosensitizer limits the production of ROS, resulting in attenuation of the antibacterial effect of a-PDT. At present, no consensus exists on the optimal setting of photosensitizers in a-PDT in periodontal practice (i.e., concentration, incubation time, etc.). Indeed, various concentrations of photosensitizers have been used for periodontal treatment of a-PDT (i.e., methylene blue: 0.005–1%; toluidine blue: 0.1–0.5%; and indocyanine green: 0.025–0.5%) [2]. Meanwhile, in most of the previous studies, the incubation time of the photosensitizers was set to 1 min, and the antibacterial efficacy of a-PDT was evaluated. A longer incubation time would increase the amounts of photosensitizers on the bacterial cells, resulting in strong antibacterial effects. However, the cytotoxicity of photosensitizers themselves has also been demonstrated in in vitro studies, and the incubation period would preferably be the minimum time required for the photosensitizers to be deposited on the target bacteria. Further in vitro and clinical studies should be conducted to determine this.

Inflammatory periodontal destruction is caused by dysbiotic polymicrobial communities, and at least 30–100 species are generally identified from a single periodontal pocket [99]. Recent research on the periodontal microbiota has clarified how the interactions between specialized community members determine an emergent overall function, which promotes or destabilizes periodontal tissue homeostasis. For example, although they are present in low numbers, keystone pathogens contribute to the emergence of dysbiotic microbiota by subverting the host immune response [100]. The appropriate wavelength of light, characteristics of the photosensitizer, and dose of light required to kill the bacteria vary with

the species, and it may be necessary to determine the species or specific group of bacteria, which will be the therapeutic target of a-PDT.

Increasing the bactericidal effect is not the only way to enhance the therapeutic efficacy of a-PDT. Some wavelengths of light can activate host cells; this is known as PBM therapy [101–103]. The combination of multiple narrow wavelengths and intensities of LED light may be used as a new light source with high antibacterial properties and low toxicity to host cells, thereby increasing the effectiveness of periodontal treatment [104]. In addition, concurrent mechanical debridement of plaque biofilms and dental calculus will increase the effectiveness of a-PDT in treating periodontal disease. Bacteria surrounded by a biofilm matrix are less active and can withstand starvation and other harsh conditions. It is difficult for photosensitizers to penetrate biofilms, and in vitro studies have reported that biofilm-formed bacteria are more resistant to a-PDT than planktonic bacteria [35,105–107]. Although clinical validation is required, repeated application of a-PDT may also be effective [108,109].

The number of clinical trials using LED-based a-PDT for periodontitis is limited. Moreover, there is also heterogeneity in the study designs, which makes it difficult to compare their results. Nevertheless, previous research suggests that a-PDT using red or blue LED light appears effective in attenuating bacteria associated with periodontal diseases. Further randomized controlled trials with a standardized application protocol should be conducted to promote the clinical use of LED-based a-PDT. Progress in this research will increase the precision and reliability of these therapies in periodontal practice. LED devices have attractive features, which lasers do not possess. Thus, LEDs have the potential to provide a new direction for light therapy in periodontics.

Author Contributions: Conceptualization, Y.T. and A.A.; investigation, C.C., A.I., N.A., Y.K., S.H. and K.T.; data curation, Y.T. and A.A.; writing—original draft preparation, Y.T. and A.A.; writing—review and editing, Y.T., A.A., K.H., C.C., A.I., N.A., Y.K., S.H., K.T., T.I. and A.A.; visualization, Y.T.; supervision, A.A., T.I. and S.A.; project administration, Y.T. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Japan Society for the Promotion of Science KAKENHI (grant number 20K09971 to A.A. and Y.T., 20K09934 to Y.T.).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: We greatly appreciate Yuichi Izumi (Southern TOHOKU Research Institute for Neuroscience, Southern TOHOKU General Hospital) for their excellent assistance.

Conflicts of Interest: The authors have no conflict of interest directly relevant to the content of this article.

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